Effects of Induced Seizures and Possible Role of Inhibitory Synaptic Plasticity in the Pathophysiology of Neurodegenerative Diseases

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ZUSAMMENFASSUNG

Die Alzheimer Krankheit (Alzheimer's disease, AD) gehört zu den häufigsten altersabhängigen Demenzerkrankungen mit mehreren Millionen Betroffenen weltweit. Sie ist durch eine schnell fortschreitende Abnahme der kognitiven Fähigkeiten und durch Neurodegeneration gekennzeichnet. Histopathologisch ist AD durch das Auftreten von amyloiden (Aβ) Plaques, Neurofibrillen und einer neuronalen Entzündung charakterisiert. Die in den Plaques vorhandenen Aβ Peptide entstehen aus der proteolytischen Verarbeitung des amyloiden Vorläufer Proteins (amyloid precursor protein, APP). Mutationen in Genen von APP oder Proteinen, die für dessen Verarbeitung verantwortlich sind, gelten als Hauptursache für die früh auftretende, familiäre Form von AD. Aus diesem Grund hat sich die Forschung lange auf die APP Verarbeitung und die Aβ Ablagerung fokussiert. Jedoch zählt die überwiegende Mehrzahl von Fällen zur sogenannten sporadischen Form von AD, dessen genaue Ursache nach wie vor unklar ist. Verschiedene Hinweise deuten darauf hin, dass sich die pathophysiologischen Mechanismen, die der familiären und sporadischen AD zugrunde liegen, unterscheiden. Verschiedene genetische, umweltbedingte und Lebensstyl-abhängige Risikofaktoren für die sporadische AD wurden identifiziert, von welchen angenommen wird, dass sie synergetisch für den Ausbruch dieser multifaktoriellen Erkrankung verantwortlich sind. Bis anhin gab es jedoch kein Tiermodel für die sporadische AD. Unsere Forschungsgruppe hat jedoch beobachtet, dass eine maternale Immunstimulation während der späten Schwangerschaft gefolgt von einer zweiten Stimulation in den adulten Nachkommen einen altersbedingten, AD-ähnlichen Phänotyp in Wildtypmäusen verursacht. Dieses sogenannte „double hit" Mausmodell gehört zu den ersten Versuchen jene spezifischen Veränderungen im Hirn zu induzieren, die es ermöglichen sollen die Mechanismen der sporadischen AD zu untersuchen.

In den letzten Jahrzehnten kamen Hinweise zum Vorschein einer Komorbidität von AD und Epilepsie. In beiden Krankheiten wurden beispielsweise Netzwerkanomalien infolge einer Dysfunktion von Interneuronen nachgewiesen, wobei diese im Falle der AD den für die AD charakteristischen Symptomen vorauszugehen scheinen. Neuronale Entzündung, welche in beiden Erkrankungen vorkommt, so vermuten einige, stellt möglicherweise ein Bindeglied zwischen den beiden Erkrankungen dar.

Das Hauptziel dieser Doktorarbeit ist den Effekt von Netzwerkanomalien in der Entwicklung der AD Pathologie und einen neuentdeckten Mechanismus der Verstärkung inhibitorischer Übertragung zu untersuchen. Letzteres könnte möglicherweise direkten Einfluss haben auf die

Pathophysiologie von Erkrankungen des zentralen Nervensystems (ZNS), wie beispielsweise die AD.

Das erste Projekt untersucht den Beitrag von Kainate (KA)-induzierten Anfällen auf die AD-ähnliche Pathologie im „double hit“ Model der sporadischen AD und dem ArcticAβ Maus Model der familiären AD. Die unilaterale Injektion von KA in den Hippocampus von adulten Mäusen ist ein gut etabliertes Model der Temporallappen Epilepsie. Die Injektion verursacht eine Läsion, welche charakterisiert ist durch den Verlust von Zellen im Hilus, CA1, und in Teilen von CA3, einer Dispersion von Körnerzellen und einer exzessiven, neuronalen Entzündung. Die ersten 24 Stunden nach der Injektion sind gekennzeichnet durch den Status epilepticus mit konvulsiven Anfällen, gefolgt von einer zweiwöchigen, anfallsfreien Latenzphase worauf spontan wiederkehrende Anfälle (spontaneous recurrent seizures, SRS) auftreten für die restliche Lebensdauer des Tieres.

Zunächst haben wir getestet, ob KA-induzierte epileptische Anfälle in pränatal immunstimulierten Mäusen die zweite Immunstimulation des „double hit“ Models ersetzen kann und eine AD-ähnliche Pathologie hervorruft. Ferner wollten wir wissen, ob das Auftreten von Anfällen ausreicht die Entwicklung einer AD-ähnliche Pathologie nach einer Immunstimulation im erwachsenen Alter zu begünstigen. Immunohistochemische Analysen der beiden Kohorten im Alter von 9 und 15 Monaten wiesen jedoch keine Anzeichen einer AD Pathologie auf. Allerdings haben sich Zweifel erhoben über den Erfolg der Immunstimulation, als nicht-epileptische Kontrollmäuse keine Anzeichen einer chronischen Entzündung nach einer Immunstimulation aufwiesen.

Gleichermassen haben wir den Effekt von KA-induzierten Anfällen auf die AD-ähnliche Pathologie im ArcticAβ Maus Model der familiären AD untersucht. Bereits vor dem Auftreten der Plaque Pathologie waren diese Mäuse sehr anfällig auf KA, weshalb fürsorglich Diazepam nach der Operation verabreicht wurde um eine unmittelbare Mortalität zu verhindern. Nichtdestotrotz, die Vulnerabilität von ArcticAβ Mäuse gegenüber Anfällen blieb über diese initiale Phase hinaus bestehen und die überwiegende Mehrheit dieser KA-injizierten AD Tiere starb in den Folgemonaten. Diejenigen ArcticAβ Mäuse, welche die 9 Monate überlebten, wiesen eine vergleichbar milde Läsion auf, wobei sich das Läsionsmuster ausschliesslich auf einen Zellverlust in CA1 beschränkte. Überraschenderweise war dieser ipsilaterale Hippocampus frei von Aβ Plaques. Der fehlende Unterschied zwischen nichtinjizierten und injizierten ArcticAβ Mäuse hinsichtlich der Ablagerung von Plaques im Neokortex liess uns schlussfolgern,

dass die KA Injektion lokale Veränderungen nach sich zieht, die die Bildung von Plaques entweder verhindert oder zumindest verzögert.

Frühere Studien haben gezeigt, dass eine Unterdrückung der adulten Neurogenese den Effekt von KA verstärkt. Ferner ist bekannt, dass viele Mausmodelle der familiären AD eine Veränderung in der adulten Neurogenese aufweisen. Aus diesem Grund haben wir die adulte Neurogenese der ArcticAβ Mäusen charakterisiert im Hinblick auf einen möglichen Erklärungsansatz für den Ursprung der Sensitivität dieser Mäuse auf KA. Im Vergleich zu Wildtypen zeigen die unreifen, adult-geborenen Neuronen von ArcticAβ Mäusen eine reduzierte dendritische Verästlung und Dornendichte (d.h. Dichte von Spines). Eine Beeinträchtigung in der Reifung solcher der adulten Neurogenese entstammenden Neurone kann zur Folge haben, dass diese inadäquate in das hippocampale Netzwerk integriert werden. Es ist bekannt, dass diese adult-geborenen Neurone in ihrer Gesamtheit einen inhibitorischen Effekt auf das Gyrus dentatus-CA3 Netzwerk ausüben und somit lässt sich vermuten, dass sie potentiell zur Sensitivität der ArcticAβ Mäuse auf KA beitragen.

Eine Fehlfunktion von Interneuronen wurde häufig beobachtet in der AD. GABAerge Interneurone orchestrieren die Aktivität von Netzwerkoszillationen und/oder kontrollieren die Entladungsrate und das Entladungsmuster von Hauptzellen. Der zweite Hauptteil dieser Doktorarbeit fokussiert auf einen neuentdeckten Mechanismus der Verstärkung inhibitorischer Übertragung in Sternzellen des Kleinhirns. Es konnte gezeigt werden, dass eine Erhöhung der mitochondrialen reaktiven Sauerstoffspezies (mitocondrial reactive oxygen species, mROS), beispielsweise infolge eines hohen metabolischen Bedarfs der Zelle, als protektiver Feedback Mechanismus fungieren kann, indem zusätzliche GABAARs an die Synapse rekrutiert werden. Interessanterweise haben diese rekrutierten Rezeptoren eine spezifische Zusammensetzung von Untereinheit, sowie eine unterschiedliche Zerfallskinetik im Vergleich zu der überwiegenden Mehrheit der ansässigen Rezeptoren. Unsere Resultate zeigten, dass eine wiederholte hochfrequente Stimulation der Inputfasern eine Wiederauferweckung (engl., un-silencing) eines bestimmten Vorrats von GABAARs und eine Rekrutierung an die Synapse zur Folge hat. Der genaue Mechanismus und die Signalkaskaden, die dieser Verstärkung der GABAergen Übertragung zugrunde liegen, sind nach wie vor unbekannt. Jedoch ist zu vermuten, dass ein Zusammenbruch eines solchen protektiven Feedbackmechanismus zu einer anomale Aktivität von Interneuronen oder sogar deren Degeneration infolge oxidativen Stresses führen könnte. Dies hätte wiederum einen grossen Einfluss auf das gesamte Netzwerk. Sollte sich bestätigen, dass dies ein allgemeiner Mechanismus ist, den man in verschieden Typen von Interneuronen

findet, bestände die Möglichkeit, dass eine fehlerhafte Wiederauferweckung von GABAergen Synapsen durch mROS Bestandteil der Pathophysiologie verschiedenster ZNS Krankheiten ist, inklusive der AD.

ABSTRACT

Alzheimer's disease is one of the most common age-related dementias affecting millions of people worldwide. Characteristic features of this disorder are progressive loss of cognitive abilities and neurodegeneration. Histopathological hallmarks of AD include formation of amyloid beta (Aβ) plaques, neurofibrillary tangles and neuroinflammation. Aβ peptides arise from proteolytic processing of amyloid precursor protein (APP). Mutations in genes encoding APP and its processing machinery cause familial early-onset AD. For a long time the focus of AD research was on APP processing or Aβ deposits. However, the vast majority of AD cases are sporadic and so far there is no clear answer to what triggers the pathology. It is likely that pathophysiological mechanisms leading to familial and sporadic AD are different. Various genetic, environmental and lifestyle risk factors have been identified for sporadic AD, suggesting that it is most likely a multifactorial disorder that develops when a number of factors converge. So far, there is no animal model of sporadic AD. However, our lab has observed that a maternal immune challenge at late gestation followed by a second immune challenge in adult offsprings triggers age-dependent development of AD-like pathology in wild-type mice. This "double hit" model is a first attempt to induce brain alterations relevant for exploring the mechanisms of sporadic AD.

In the last decade evidence of comorbidity of AD and epilepsy has emerged. Network abnormalities due to interneuron dysfunction are common in both conditions and in case of AD they appear prior the onset of typical symptoms. Neuroinflammation, which also occurs in both diseases, is a possible link between the two conditions.

The overall goal of this thesis is to study the effects of network abnormalities on development of AD pathology and to investigate a newly described strengthening mechanism of inhibitory transmission, which could potentially be implicated in the pathophysiology of CNS diseases, including AD.

The first project focused on the contribution of kainic acid (KA)-induced seizure activity to AD-like pathology in the sporadic "double hit" and familial ArcticAβ AD mouse models. Unilateral intrahippocampal injection of KA in adult mice is a well-established model of temporal lobe epilepsy. The injection causes a lesion characterized by the loss of hilar cells, CA1 and partly CA3, dispersion of granule cell layer and extensive neuroinflammation. During the first 24 hours post-injection, status epilepticus with convulsive seizures occur, followed by a two-week long

seizure-free latent phase after which spontaneous recurrent seizures (SRS) arise for the life-time of the animal.

First, we tested whether KA-induced epileptic seizures in prenatally immune-challenged mice could substitute the second immune insult and lead to AD-like pathology.

Our next question was to understand whether a brain with already induced seizure activity can develop AD-like pathology following an immune insult in adulthood. Immunohistochemical analysis of both cohorts of mice did not show any signs of AD pathology at the age of 9 and 15 months. However, control non-epileptic mice did not exhibit signs of chronic inflammation after the immune challenge, raising doubts about its effectivity.

We went on to analyze the effects of KA induced seizures on AD-like pathology in familial AD ArcticAβ mice. Already at the pre-plaque stage, these mice were highly sensitive to KA and required application of diazepam after surgery to prevent immediate mortality. Nevertheless the majority of KA-treated ArcticAβ mice died during the following months after injection. A group of mice that survived till 9 months had a "mild" KA lesion phenotype, with degeneration only in the CA1 region. Unexpectedly, the ipsilateral hippocampus was devoid of Aβ plaques. As there was no difference in plaque load in the neocortex of these mice compared to control, we concluded that KA injection induces changes that prevent or delay plaque formation.

Suppression of adult neurogenesis was shown to enhance the effects of KA. Many FAD mice are reported to have altered adult neurogenesis. We attempted to explain the sensitivity of ArcticAβ to KA by characterizing adult neurogenesis in the hippocampus. Immature adult born neurons showed decreased dendritic arborization and decreased spine density. Impaired maturation of adult-born neurons can result in their inadequate integration within the hippocampal networks. It is known that adult-born neurons exert net inhibitory effect on dentate gyrus-CA3 network in the hippocampus; therefore, this could contribute to the sensitivity of ArcticAβ mice to KA.

Dysfunction of interneurons is frequently reported in AD. GABAergic interneurons orchestrate network oscillatory activity or/and control firing rate and patterns of principal cells. The second major part of this thesis focused on a unique newly described inhibitory strengthening mechanism in cerebellar stellate interneurons. It has been shown that an increase of mitochondrial reactive oxygen species (mROS), for example due to high metabolic demand in the cell, can function as a protective feedback mechanism by recruiting more $GABA_A$Rs to the synapses. Interestingly, the recruited receptors have distinct subunit composition and decay kinetics in comparison to the resident predominant receptors. Here, we show that repeated high

frequency stimulation of the input fibers leads to un-silencing of a distinct pool of GABAARs by recruiting them to the synapse. The exact mechanisms and signaling pathways that underlie this strengthening of GABAergic transmission are still to be uncovered. However, the breakdown of such a protective internal feedback mechanism could trigger abnormal firing of interneurons, or even their degeneration due to oxidative stress. This, in turn, would have a major impact on entire networks. If proved to be a common mechanism in many other types of interneurons, failed un-silencing of GABAergic synapses by mROS might contribute to the pathophysiology of many CNS disorder, including AD.

I. GENERAL INTRODUCTION

Alzheimer's disease (AD)

First described at a conference over a century ago in Tübingen, Germany, by the psychiatrist and neuropathologist Alois Alzheimer, Alzheimer's disease (AD) is now recognized as the most common age-related dementia affecting millions of people world-wide (Ferri et al., 2005, Prince et al., 2013). Typically, memory deficits are the first cognitive symptoms of developing AD. With progression of the disease an array of cognitive abilities gradually declines and patients can exhibit problems with spatial orientation, troubles with executing daily tasks, such as handling money or getting dressed. At later stages memory continues to deteriorate. Patients have problems communicating and recognizing family and friends have changes in personality and behavior may experience hallucinations, delusions, and many are reported to have seizures. At latest AD stages, patients have other physiological symptoms, such as difficulties controlling bowel and bladder, difficulties swallowing, increased sleeping, weight loss. (National Institute of Ageing, NIH, 2017, https://www.nia.nih.gov/alzheimers/topics/symptoms) Post-mortem brain tissue of AD patients is characterized by severe neurodegeneration, extracellular accumulation of amyloid-β (Aβ) peptide plaques, intracellular formation of neurofibrillary tangles (NFTs), that consist of hyperphosphorylated tau protein and neuroinflammation (Castellani et al., 2010, Serrano-Pozo et al., 2011).

APP processing and amyloid cascade hypothesis

Most AD cases are sporadic and about 1% of AD cases are familial, with an identified underlying genetic cause and those are mostly early-onset. About two hundred autosomal dominant mutations in amyloid precursor protein (APP) and presenilin 1 and 2 genes (PS1 and PS2), which contribute to proteolytic cleavage of Aβ, are associated with early-onset familial forms of AD (Cruts et al., 2012); Alzheimer Disease and Frontotemporal Dementia Mutation Database http://www.molgen.vib-ua.be/ADMutations). The gene encoding APP belongs to the class of housekeeping genes (Salbaum et al., 1988, Izumi et al., 1992, Quitschke and Goldgaber, 1992). Although the functions of APP are still being investigated, it was shown to play a role in development of CNS, neurite outgrowth and synaptogenesis (Muller et al., 2017). Neurons produce large amounts of APP, which is cleaved by α, β and γ-secretases enzyme complexes. In the non-amyloidogenic pathway, cleavage of APP by α-secretase occurs within the Aβ domain

and consequently prohibits Aβ peptide production. APP is cleaved by α-secretase (ADAM9, ADAM10 and ADAM17) to generate two fragments; a transmembrane 83 amino acid C-terminal fragment (C83) and an N-terminal ectodomain (sAPPα) that is released into the extracellular space (Figure 1) (Allinson et al., 2003). sAPPα can be further cleaved by γ-secretase into fragments that are thought to be irrelevant for the progression of the disease. On the other hand, the amyloidogenic pathway leads to generation of neurotoxic Aβ species. The first proteolysis step is mediated by β-secretase (BACE1), which releases a large N-terminal ectodomain (sAPPβ) into the extracellular space and leaves a 99-amino acid C terminal fragment (C99) remaining in the membrane (Sinha et al., 1999, Sinha and Lieberburg, 1999, Vassar et al., 1999). Aβ is released when C99 is further cleaved by γ-secretase between residues 38 and 43. γ-secretase is a complex of enzymes consisting of presenilin 1 or 2 (PS1 and PS2), nicastrin, anterior pharynx defective (APH-1) and presenilin enhancer 2 (PEN2) (Wolfe et al., 1999, Yu et al., 2000, Levitan et al., 2001, Francis et al., 2002, Steiner et al., 2002).

Aβ peptide is highly resistant to proteolytic degradation. Aβ isoforms consist of 37-43 amino acids, among which $A\beta_{1-40}$ and $A\beta_{1-42}$ are the most common. Prone to β-sheet type folding, hydrophobic $A\beta_{1-42}$ is the most toxic isoform, that predominantly forms the core of the amyloid plaques. Location of familial mutations within APP and APP processing machinery genes and $A\beta_{1-42}$ toxicity lead to the emergence of amyloid cascade hypothesis (Hardy and Higgins, 1992). The hypothesis centers around the generation of toxic Aβ species being a major cause of the disease.

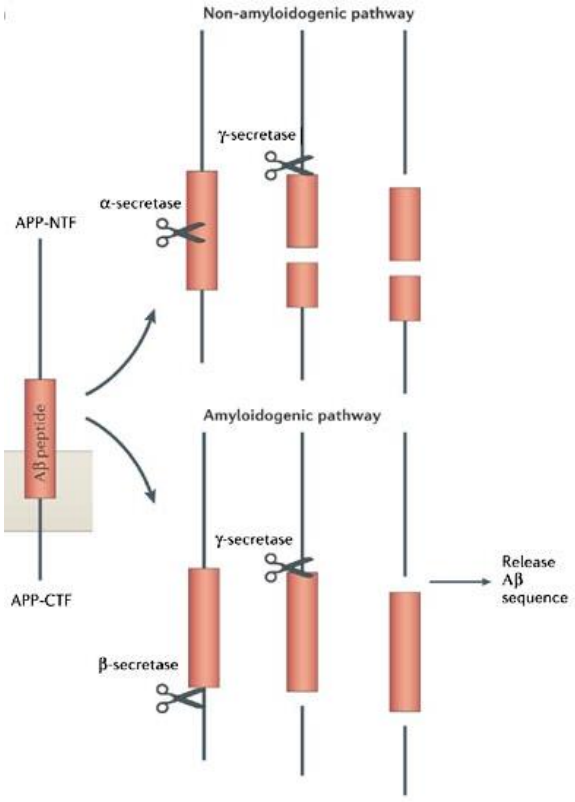

Figure 1. APP processing. Amyloid precursor protein (APP) is cleaved by multiple secretases. In non-amyloidogenic pathway Aβ region of APP is cleaved in the middle by α-secretase and two soluble APP fragments are released. The amyloidogenic pathway results in releases of Aβ peptides after APP has been cleaved by β- and γ-secretases (Van Dam and De Deyn, 2006).

Cholinergic hypothesis

The cholinergic system in the brain is involved in neuromodulation and some deterministic tasks that affect learning and memory (Picciotto et al., 2012, Sarter et al., 2014). The "cholinergic hypothesis" was the first approach to describe AD pathophysiology. It was defined in the 70s after correlating mental state of the patients with post-mortem histopathological and biochemical alterations. These screenings identified selective damage to cholinergic neurons and axons in the hippocampus, frontal cortex, amygdala, nucleus basalis and medial septum, regions involved in learning and memory, conscious awareness, and attention. Findings included decreased activity of choline acetyltransferase (ChAT) (Bowen et al., 1976, Davies and Maloney, 1976, Perry et al., 1977), reduced choline uptake (Rylett et al., 1983), decrease in ACh release (Nilsson et al., 1986), reduced levels of nicotinic and muscarinic M2 receptors (Whitehouse et al., 1988, Nordberg et al., 1992a), at presynaptic terminals and degeneration of ACh producing cells in the

basal forebrain and medial septum (Whitehouse et al., 1982, Francis et al., 1999). Postsynaptic muscarinic receptors appeared to be less affected (Whitehouse et al., 1988, Nordberg et al., 1992b),.

Alterations in cholinergic system have been associated with development of histopathological hallmarks of AD. For instance, the non-selective muscarinic antagonist scolopamine can induce cognitive impairment and decrease the activity of α-secretase, leading to increased production of Aβ peptide (Liskowsky and Schliebs, 2006). As for tau pathology, in vitro studies have linked activation of M1 muscarinic receptors to decreased Tau phosphorylation (Sadot et al., 1996). Muscarinic M1 receptors were shown to affect the level of activated major Tau kinase - GSK-3β (Medeiros et al., 2011).

Major AD therapy drugs are competitive inhibitors of ACh esterase – the enzyme that breaks down ACh. These drugs promote temporary cognitive improvement, but are not effective in all patients (Francis et al., 1999, Cummings et al., 2014), which raises questions about the causal role of cholinergic system defects in development of sporadic AD (Craig et al., 2011).

Role of long-range brainstem projections in AD

It has been observed that AD-related pathology first appears at specific sites in the human brain and further spreads to other regions in a specific manner (Braak and Braak, 1991, Nelson, 2007, Duyckaerts et al., 2009a, Duyckaerts et al., 2009b, Nelson et al., 2011, Serrano-Pozo et al., 2011, Hyman et al., 2012, Montine et al., 2012). The first observable AD-related alteration is hyperphosphorylation and aggregation of cytoskeletal protein tau. This pathology can first be seen in the brainstem nuclei that project to the cerebral cortex. Serotonergic upper raphe nuclei, the noradrenergic locus coeruleus and cholinergic magnocellular nuclei are among them. Those nuclei contain neurons with longest axons in the CNS that are prone to increased mitochondrial oxidative stress during aging (Sanchez-Padilla et al., 2014). Oxidative stress can directly induce tau aggregation (Schweers et al., 1995), which can lead to formation of PHFs as well as early NFT-induced neurodegeneration (Grudzien et al., 2007). The density of Aβ plaques is high in the projection areas of these brainstem nuclei, such as the olfactory bulb, striatum, neocortex, cerebellar cortex, tectum and lower brainstem and minimal in brainstem nuclei. It was suggested that Aβ is released from the non-junctional varicosities of long range axons of brainstem neurons with tau pathology (Braak and Del Tredici, 2013).

Synaptic dysfunction in AD

It was recognized already over two decades ago, that severity of AD related cognitive impairments correlates with the degree of synaptic loss rather than with Aβ plaque load or neurofibrillary tangles (Hamos et al., 1989, DeKosky and Scheff, 1990, Robinson et al., 2014). Synaptic dysfunction occurs before plaque pathology (Oddo et al., 2003). Almost 30% decrease of cortical synapses and a reduction in pre-and postsynapse-associated proteins was reported in patients with AD (Reddy and Beal, 2008). Soluble Aβ monomers can form low molecular weight (LMW) oligomers (dimers or trimers), or high molecular weight (HMW) oligomers and fibrils or protofibrils, that deposit in plaques (Larson and Lesne, 2012). Notably, Aβ oligomers but not fibrils have been associated with synaptic alterations (Ferreira and Klein, 2011). High concentrations of soluble Aβ oligomers are found in cerebrospinal fluid (CSF) of AD patients. Soluble Aβ oligomers were found associated with synapses in the vicinity of Aβ plaques in brains of AD patients, but not in asymptomatic controls (Herskovits et al., 2013, Bilousova et al., 2016). Soluble oligomers contribute to cognitive impairments in AD patients by inhibiting long-term potentiation (LTP) and facilitating long-term depression (LTD) at excitatory synapses. There is evidence indicating that specifically LMW oligomers contribute to synaptic loss, which ultimately leads to permanent memory loss. HMW oligomers appear to be less toxic, but were shown to be capable of dissociating into LMW oligomers (Wang et al., 2016, Yang et al., 2017),

Already before the occurrence of classical AD pathological hallmarks, accumulation of Aβ is observed intracellularly and shown to be toxic (Wirths et al., 2012, Youmans et al., 2012, Iulita et al., 2014, Yang et al., 2017). Concentration of HMW and LMW oligomers increases in brain interstitial fluid with age. Evidently, at later stages Aβ oligomers can derive from plaque halo and accumulate at excitatory synapses at both pre- and postsynaptic sites (Heras-Sandoval et al., 2012, Takeda et al., 2013, Pickett et al., 2016). It is suggested that Aβ oligomers exert their toxic effect by binding to postsynaptic receptors (Um et al., 2013, Jarosz-Griffiths et al., 2016). Aβ oligomers can bind to both ionotropic and metabotropic receptors. Through the recruitment of Nedd4-1ubiquitin ligaze, Aβ oligomers induce reduction of surface AMPA receptors, spine density and synaptic strength. Aβ oligomers were also shown to be able to induce LTD through NMDA receptors and PKCα/PICK1 (protein kinase C α/ protein interacting with C kinase 1) dependent pathways that lead to the reduction of AMPA receptors in the synapses (Alfonso et al., 2014, Alfonso et al., 2016, Reinders et al., 2016, Rodrigues et al., 2016).

In conclusion, in the recent decade a progress in accepting a crucial role of oligomers in AD pathophysiology has been made. With advance in PET imaging it is now possible to measure

synaptic density in human patients which would contribute to both early diagnosis and research in the field (Finnema et al., 2016). Reducing the presence or availability of Aβ oligomers is a promising therapeutic goal.

Familial AD mouse models

Overexpressing mutant human APP is the most common strategy to generate AD transgenic mouse models. The Aβ sequence of wildtype mice and humans differ and due to the fact that mice do not develop AD, transgenic mouse lines have to be used (Dyrks et al., 1993). Mice that overexpress wild-type human APP have only mild neuropathological changes without deposition of Aβ plaques, suggesting that wildtype APP overexpression alone may not trigger the human disease efficiently in mice (Buxbaum et al., 1993, Lamb et al., 1999, Mucke et al., 2000). On the other hand, expression of human APP or presenilin with FAD mutations leads to age-dependent development and maturation of Aβ plaques in the brain (Platt et al., 2013). The age of the onset of Aβ plaques deposition varies between mouse models, depending on specific mutations. In FAD mouse models plaques usually start to form after the age of 6-7 months. There are some exceptions, for instance 5xFAD mouse model, which combines 5 mutations in APP and presenelin and develops extensive plaque pathology by 2 months of age (Oakley et al., 2006). Cognitive impairments are also present in many transgenic mice (Games et al., 1995, Hsiao et al., 1996, Sturchler-Pierrat et al., 1997). Such hallmarks as synaptic loss, dystrophic neurites, reactive astrocytes and activated microglia usually follow and accompany the formation of Aβ plaques.

In our study we used ArcticAβ transgenic FAD mouse line that expresses human APP695 with Swedish (Swe; KM670/671NL) and Arctic (E693G) mutations under the prion protein promoter (PrP) (Knobloch et al., 2007). Swedish mutation is a double mutation, the only one known, that is immediately adjacent to the β-secretase site in APP. This mutation runs in two large Swedish families which were found to be genealogically connected (Mullan, 1992, Mullan et al., 1992). In vitro studies showed that this mutation increases the production and secretion of $A\beta_{40}$ and $A\beta_{42}$, without affecting the $A\beta_{40}/A\beta_{42}$ ratio. (Citron et al., 1992, Cai et al., 1993, Citron et al., 1994, Johnston et al., 1994, Citron et al., 1996, Scheuner et al., 1996, Nilsberth et al., 2001). The Arctic mutation found in a family from Northern Sweden is one of a few mutations located in Aβ sequence of APP. The biological effect of the Arctic mutation comprises an increased propensity and faster rate $A\beta_{40}$ protofibrils formation in comparison to wild-type $A\beta_{40}$ (Nilsberth et al., 2001). Along with other mutations found in Aβ sequence, the Arctic mutation causes cerebral

amyloid angiopathy (CAA), probably due to inefficient clearance of Aβ across the blood-brain barrier (BBB) (Monro et al., 2002, Lashuel et al., 2003).

ArcticAβ mice show increased Aβ production, which is followed by the formation of intracellular punctate Aβ deposits that coincide with onset of behavioral deficits. Impaired working memory in ArcticAβ mice was first observed at the age of 6 months, preceding the formation of extracellular Aβ plaques and the development of CAA. This finding suggests an important role of early Aβ deposits in the pathogenesis of AD.

Sporadic AD mouse model

Familial AD (FAD) mutations are responsible for early-onset relatively rare cases of AD. Most AD cases occur sporadically and later in life (Herrup, 2010). Interestingly, genome wide association studies did not identify a correlation between polymorphisms in genes encoding APP and APP processing enzymes and the incidence of late-onset AD. Except a well-known risk factor for AD - Apolipoprotein E ε4 allele (APOE4) (Corder et al., 1993), genome-wide association studies have shown correlation between polymorphisms in genes of the innate immune system and late-onset disease incidence. (Harold et al., 2009, Lambert et al., 2009). Therefore, the underlying pathogenesis could differ between the familial and sporadic forms of AD.

Animal models used in AD research are based on FAD mutations, and considering the epidemiology of AD, the field would immensely benefit from reliable models of sporadic AD.

Large amount of evidence link immune system function to AD. The exact role is still debated. Activation of innate immunity was implicated in triggering AD, being beneficial, or considered to be a disease by-product (Wyss-Coray, 2006). For instance, it was shown that exogenously applied interleukin (IL) 1 increases APP levels and Aβ peptides production *in vitro* (Sheng et al., 1996). Tumor necrosis factor alpha (TNFα) has also been shown to increase Aβ peptides production *in vitro* and *in vivo* (Li et al., 2004, Medeiros et al., 2007, Yamamoto et al., 2007). Elevation of TNF receptors in humans has been associated with increased β-secretase activity and increase levels of Aβ in CSF, which is a predictor of mild cognitive impairment (MCI) patients developing into AD (Buchhave et al., 2010). Interestingly, people who suffer from rheumatoid arthritis and take non-steroidal anti-inflammatory drugs (NSAIDs) have lower risk of developing AD (McGeer and McGeer, 1996, McGeer et al., 1996, Stewart et al., 1997). These

are just some examples of the numerous findings that implicate the immune system in the development of AD pathology.

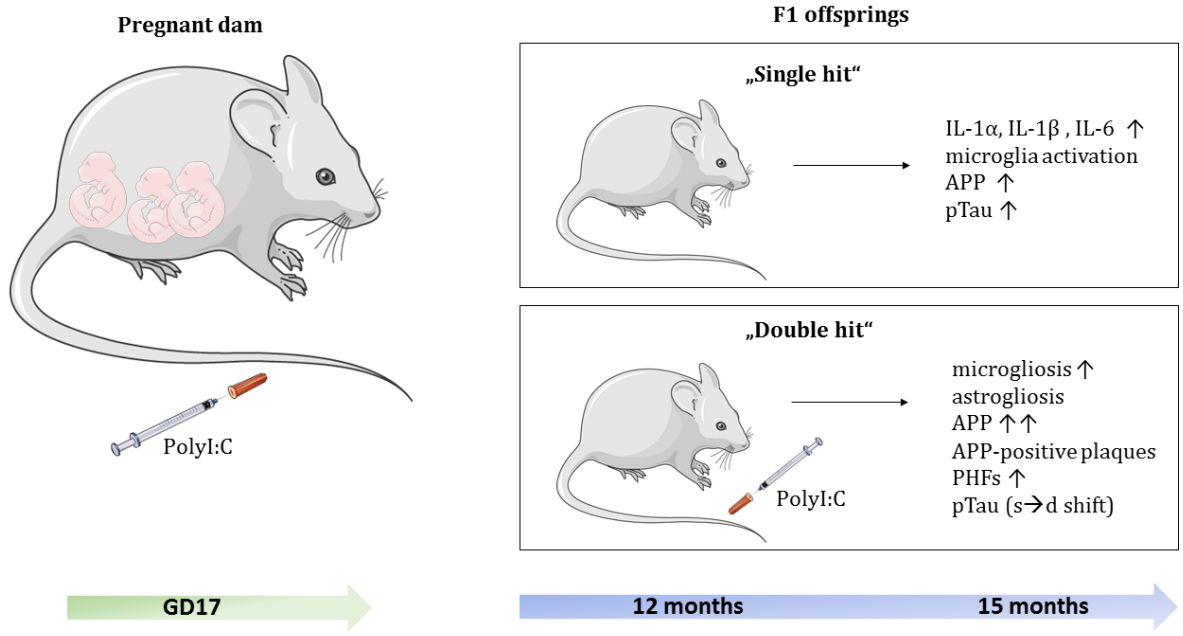

Figure 2: "Double-hit" mouse model for sporadic AD. Pregnant dams (GD17) were i.v. injected with 5 mg/kg of PolyI:C at gestational day 17 (GD17). First generation (F1) offsprings exposed only to prenatal PolyI:C ("single hit") develop AD-related changes with age, including central increased levels of pro-inflammatory cytokines (IL-1a and -1b and IL-6), activation of microglia and increased levels of APP and pTau. A second exposure of F1 to PolyI:C (5 mg/kg) at the age of 12 months ("double hit") exacerbates the phenotype detected at the age of 15 months, resulting in more prominent micro- and astrogliosis, further APP elevation and formation of APP-positive plaques as well as increased formation of paired helical filaments (PHFs) and a somatodendritic shift of pTau. Illustrations taken from http://smart.servier.com/.

The immunostimulant polyinosinic:polycytidylic acid (PolyI:C), which mimics viral infections by interacting with toll-like receptor 3 (TLR3), expressed by B-cells, macrophages and dendritic cells, is widely used in immune research. Structurally it resembles double-stranded RNA, present in some viruses. It was previously shown that systemic administration PolyI:C to pregnant mice during late gestation causes a long-lasting elevation of inflammatory cytokines, memory impairments and accelerated deposition of aggregated proteins in the offspring (Meyer et al., 2006, Meyer et al., 2008, Knuesel et al., 2009). Exposure of the offspring to a second PolyI:C challenge in adulthood triggered development of AD-like pathology during ageing (Krstic et al., 2012). The same systemic immune challenge in adult transgenic AD mice also exacerbated age-related AD-like pathology, suggesting a causal role of chronic activation of the immune system in this process.

Comorbidity of AD and epilepsy

AD and epilepsy are largely viewed as separate syndromes belonging to distinct medical subspecialities. In the recent years, there has been mounting evidence for comorbidity of these two diseases. For instance, patients suffering from AD have 6 to 10- fold higher risks to develop seizures and epilepsy compared to the healthy individuals of the same age (Hesdorffer et al., 1996a, Amatniek et al., 2006, Scarmeas et al., 2009). Interestingly, epileptic activity in some cases was shown to occur at the early "pre-degenerative" disease stages in AD patients and is associated with cases of more severe dementia (Hesdorffer et al., 1996b, Mendez and Lim, 2003, Amatniek et al., 2006, Scarmeas et al., 2009, Noebels, 2011). Moreover, seizures are suggested to contribute to the progression of AD (Vossel et al., 2013). Interestingly, even in people with identified genetic risks of developing AD, neurological examination performed decades before the symptoms might occur reveal activity disturbances in specific networks during memory-related tasks (Mondadori et al., 2006, Bateman et al., 2012, Reiman et al., 2012) From the side of epilepsy research, some epilepsy patients often have recurrent memory disturbances that closely resemble clinical features of early stages of AD (Jokeit and Ebner, 1999, Sinforiani et al., 2003, Lozsadi et al., 2008, Ito et al., 2009a). In addition, epileptic patients have increased incidence of senile plaques (Mackenzie and Miller, 1994, Gouras et al., 1997). This observation suggests that both epileptogenesis and early development of AD pathology might have common pathophysiology and that recurrent seizures might aggravate AD pathology and vice-versa.

Seizures in AD mouse models

For logistics and ethics reasons invasive EEG monitoring in human patients is not always feasible. This is one of the reasons for the absence of systemic large scale studies describing the incidence of seizures in human AD patients. More systemic, lengthy and invasive monitoring is possible to perform in animal models. A number of FAD transgenic mice that express human mutant APP show seizures prior the development of AD pathology hallmarks, such as Aβ plaques (Palop et al., 2007, Chin, 2008). Alterations on synaptic and molecular level can affect overall network function, which in turn, closing the vicious circle, can affect individual synapses and molecules. For instance, hAPPJ20 line, that combines Swedish double and Indiana mutations, has epileptiform activity such as spikes and sharp waves. Spontaneous epileptiform discharges occur in hAPPJ20 mice during reduced gamma oscillatory activity (20-80Hz), which is abnormal in this mouse line due to impairments in interneurons that underlie the generation of

this rhythm (Verret et al., 2012a). Overexcitability in the network may increase Aβ production. Excessive Aβ peptide production affects synaptic plasticity that may lead to cognitive deficits.
It was suggested that seizures could contribute to the cognitive deficits that occur prior amyloid deposition. Those deficits comprise impairments in spatial memory and anxiety (Palop et al., 2003, Palop et al., 2007). Interestingly, inhibiting seizures reduces these deficits (Roberson et al., 2011, Sanchez et al., 2012). Early seizure offset in transgenic AD mice suggests the importance of oligomeric Aβ species in their development. When seizures become evident, the absence of extensive neuronal loss in the AD mouse models underscores that in this case neurodegeneration is not a prerequisite for epilepsy in this disorder. In addition, hAPP mice have circuit reorganization typically induced by epileptiform activity. This includes upregulation of neuropeptide Y in GABAergic interneurons and the hippocampal mossy fiber pathway (Palop et al., 2007, Roberson et al., 2007).

Epileptic activity and network disturbances are frequently seen in FAD mouse models. The occurrence of those abnormalities prior the development of plaque pathology suggests a possible crucial role of epileptic-like activity in the development of AD pathology.

Temporal lobe epilepsy and kainic acid mouse model

Unilateral intrahippocampal injection of KA in adult mice causes a local lesion with progressive neurodegeneration of CA1 pyramidal cells and hilar cells, granule cells dispersion, severe astrogliosis and persistent microglial activation in the affected hippocampus. Life-long non-convulsive focal spontaneous recurrent seizures (SRS) appear after a two-week long latent phase (Bouilleret et al., 2000, Riban et al., 2002, Arabadzisz et al., 2005, Duveau et al., 2011b). Like in human TLE patients, SRS in mice rarely generalize and tend to remain focal. Seizures in AD are rarely detected with cortical EEGs (Noebels, 2011), making the KA mouse model optimal for investigating the link between AD and epilepsy.

Focal inflammatory processes commonly occur in TLE and other epilepsies. Among them are enhanced production of IL-1β and other cytokines by glial cells. This promotes neuronal hyperexcitability and neurodegeneration through the activation of a number of signaling cascades via TLRs (Friedman and Dingledine, 2011, Vezzani et al., 2011a, Vezzani et al., 2011b). Focal seizures cause local inflammation that promotes infiltration of brain parenchyma by macrophages and lymphocytes from periphery. Prevention of this infiltration reduces SRS in

mice (Fabene et al., 2008). PolyI:C or lipopolysaccharide (LPS) (bacterial toxin) challenge during development was shown to influence mouse susceptibility to epileptogenic substances in adulthood, initially acting through TLRs. Inflammation, as an undeniable companion of many epilepsies could represent a link through which abnormal neuronal excitability could influence AD-like pathology.

Neurogenesis in AD and epilepsy

The birth of neurons after the initial early development continues in adult brain in specific niches, namely subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SZ) (Kempermann et al., 2015). During human life around 35% of the DG neurons are replaced, which is more than three times more than in rodents (Ninkovic and Gotz, 2007, Imayoshi et al., 2008). Adult hippocampal neurogenesis plays a role in synaptic plasticity, learning and memory, and pattern separation. Cognitive deficits in AD may be, at least partly, due to impairments in adult neurogenesis (Demars et al., 2010, Lazarov and Marr, 2010, Lazarov et al., 2010, Demars et al., 2013).

Adult neurogenesis seems to be almost always affected in one way or another in AD in both humans and rodents. There are conflicting results among studies analyzing neurogenesis in AD patients, showing depletion or increase (Nagy et al., 1997, Boekhoorn et al., 2006, Ziabreva et al., 2006). Analysis of neurogenesis in different FAD mouse models varies from line to line, but in many cases neurogenesis is negatively affected (Wang et al., 2004 (Wang et al., 2004, Donovan et al., 2006, Taniuchi et al., 2007, Zhang et al., 2007, Rodriguez et al., 2008). Some animal models show increased proliferation, but decreased survival of the newborn neurons (Chevallier et al., 2005, Yu et al., 2009). Neurogenesis in hAPPJ20 mice is characterized by accelerated early development, but impaired maturation of adult-born granule cells. This was linked to increased GABAergic input to these cells and to further synaptic rewiring (Sun et al., 2009). A number of other studies linked AD-related disturbances of GABA-mediated inhibition to changes in adult neurogenesis. (Ge et al., 2007, Song et al., 2012, Wang et al., 2014).

Recent studies revealed a role adult neurogenesis plays in seizure susceptibility in multiple epileptic mouse models. Suppression of neurogenesis can increase the acute effect of KA in mice (Iyengar et al., 2015). Adult-born neurons have a net inhibitory effect on the DG-CA3 network (Acsady et al., 1998). ArcticAβ mice that share hAPP Swedish mutation with hAPPJ20

are likely to have disturbances in adult neurogenesis. Depending on the alteration, this at least partially, could explain sensitivity of these animals to KA.

Mechanisms of GABAergic synaptic strengthening

Glutamate and γ-Aminobutyric acid (GABA) mediate excitatory and inhibitory neurotransmission, respectively. Glutamate is converted into GABA by glutamic acid decarboxylase (GAD), which is located at GABAergic axon terminals. Vesicular glutamate transporter (VGluT) and vesicular GABA transporter (VGAT) are required for packing of respectively glutamate or GABA into synaptic vesicles, for further exocytosis and neurotransmission. At the postsynapse glutamate activates ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite receptors, that mediate fast excitatory transmission and N-methyl-D-aspartate (NMDA) receptors that have slower kinetics and play a central role in the synaptic plasticity (Luscher and Malenka, 2012). Glutamate also activates G-protein coupled metabotropic glutamate receptors (mGluRs) that also play a role in synaptic plasticity at both post- and presynapse.

At the postsynaptic membrane, GABA activates $GABA_A$ and $GABA_B$ receptors. $GABA_B$ are metabotropic inhibitory receptors that are G-protein coupled to potassium channels. Upon activation they "bring" neuron closer to the equilibrium potential of K^+, thus inhibiting action potential firing (Pin and Bettler, 2016). $GABA_A$Rs are Cys-loop ligand-gated chloride ion channels that mediate most of fast inhibitory transmission in the brain. $GABA_A$Rs form pentameric assemblies. So far 19 genes encoding different $GABA_A$Rs subunits in mammals have been identified (α1–α6, β1–β3, γ1–γ3, δ, ε, π, τ, ρ1–ρ3) (Olsen and Sieghart, 2009). Most frequent $GABA_A$ receptor subunit arrangement in the CNS is 2α/2β/γ. Many distinct combinations of $GABA_A$ receptors exist throughout the brain and they differ in their kinetics, pharmacological properties and expression distributions (Fritschy and Panzanelli, 2014). Postsynaptic or extrasynaptic localization of $GABA_A$ receptors determines the form of inhibition they mediate. Postsynaptic receptors usually contain α1, α2 or α3 along with β variants and γ2 and mediate fast, phasic currents upon presynaptic GABA release; extrasynaptic receptors usually contain α4, α5 or α6, two of the β2 subunits and δ subunit and mediate tonic (persistent) inhibition by ambient GABA. This consistent pattern suggests that subcellular targeting of $GABA_A$Rs can be subunit specific (Belelli et al., 2009, Brickley and Mody, 2012). Strength of GABAergic transmission is modulated by the network activity. Both post- and extrasynaptic receptors are regulated by posttranslational mechanisms through influencing receptor biophysical

properties, trafficking and synaptic confinement. (Luscher et al., 2011, Connelly et al., 2013). Scaffolding proteins such as gephyrin play a major role in postsynaptic targeting of $GABA_A$Rs. Gephyrin interacts with many proteins and thus contains numerous phosphorylation and posttranslational modification sites, providing a hub for regulation of postsynaptic $GABA_A$Rs by various factors (Fritschy et al., 2008) (Tyagarajan and Fritschy, 2014). $GABA_A$R-associated protein (GABARAP) is another regulator of $GABA_A$Rs mobility. GABARAP is involved in exocytosis of $GABA_A$Rs as a result of potentiation of glutamatergic synapses (Marsden, 2007). There are many open questions about the exact action of GABARAP, since multiple isoforms of it exist. GABARAP-KO mice show normally clustered $GABA_A$Rs. (O'Sullivan et al., 2005). It was suggested that GABARAP is not solely regulating $GABA_A$R trafficking, but forms complexes with other proteins to exert its function (Mizokami et al., 2007).

Dysregulation of mechanisms underlying the balance between neuronal excitation and inhibition and activity-dependent synaptic strengthening could be crucial in pathogenesis of a wide array of neurological diseases including epilepsy and AD.

Neuronal excitation and energy metabolism are tightly linked. The generation of mROS at the electron transport chain in the inner mitochondrial membrane can be viewed as a reflection of the intensity of cellular metabolism. Although mROS overproduction and accumulation leads to oxidative stress and is damaging for the cells, lower concentrations of mROS can be beneficial. For instance, mROS can be viewed as a signaling molecule involved in adaptations to hypoxia, regulation of autophagy, cell differentiation and aging (Sena and Chandel, 2012).

Cerebellar stellate cells are a subtype of GABAergic interneurons in the cerebellar molecular layer that innervate Purkinje cells dendrites. Stellate cells receive stable inhibitory transmission, which makes them an ideal cell type for obtaining reliable intracellular recordings of high amplitude, high frequency GABAergic synaptic events. In young adult cerebellum, stellate cells GABAergic synapses contain $GABA_A$ receptors assembled mainly with α1, β2 and γ2 subunit variants. Pharmacological inhibition of ATP production in the mitochondria to increase mROS was shown to selectively increase the frequency of miniature IPSCs of smaller amplitude and slower decay kinetics in comparison to control conditions, as seen in whole cell patch clamp recordings. Interestingly, slow decay kinetics of newly occurring inhibitory postsynaptic events matched kinetic properties of $GABA_A$ receptors containing the α3 subunit, which are expressed at low levels in stellate cells. The mROS-dependent recruitment of these receptors was demonstrated by using α3 subunit knock-out mice. Therefore, this data unravelled the existence of a molecular pathways triggered by mROS that selectively recruit extrasynaptic receptors to

postsynaptic sites in order to strengthen GABAergic transmission. This is the first study to link mROS to signaling the metabolic state of the cell to GABAergic transmission (Accardi, 2014). Furthermore, in cerebellar granule cells, extracellular insulin application was shown to strengthen inhibitory synapses by elevating mROS, leading to recruitment of α6 subunit-containing $GABA_A$Rs (Accardi et al., 2015), suggesting that this mechanism, involving $GABA_A$Rs that do not reside in postsynaptic densities might be universal.

Interneurons are important regulators of network rhythmicity and excitability of principal neurons. Impairments in interneuron firing or synaptic function and plasticity could underlie changes in synchrony and excitability of networks. mROS induced plasticity was already observed in two cell types in the cerebellum. It still remains to be researched if this plasticity is a universal way for many other neuronal types Dysfunction of mROS-induced strengthening could lead to network abnormalities and excessive oxidative stress, which could affect neuronal survival. mROS induced plasticity related pathways could be a promising candidates for search of novel therapeutic targets.

II. AIM OF THE THESIS

The central objective of this thesis is to contribute to the understanding of the neurodegenerative disorders by exploring a wide scope of mechanisms that could underlie them. On the network scale my thesis is focused on how epileptic activity contributes to Alzheimer's disease pathology. On a synaptic scale this work further investigates recently described inhibitory plasticity mechanisms, which are regulated by activity and metabolic state of a neuron. A breakdown of this plasticity mechanism can lead to increased network excitability which may in turn contribute to the development of a neurodegenerative disorder.

Study I. Effects of acquired seizures on AD pathology in sporadic and familial mouse models

In this project we investigate the role of epileptic activity in the development of AD by inducing epileptic-like pathology in mice predisposed to AD. To do this, we combine intrahippocampal kainic acid (KA) injection - a model widely used to mimic TLE with AD mouse models.

Sporadic AD

- Can induction of seizures in AD-susceptible brain trigger AD pathology reminiscent of the one induced by the second PolyI:C hit?
- Are chronic seizures a predisposition for developing AD-like pathology after a PolyI:C-induced immune challenge?

To address these questions pregnant WT dams were injected with PolyI:C at gestational day 17 (GD17). The offsprings were taken into experiments at the age of 3 months and were injected intrahippocampally with KA. To answer the second question WT mice were injected with KA at 3 months of age and 1 month later they were intravenously injected with Poly:C. In both cases at the age of 9 and 15 months their brain tissue was collected and immunohistochemically analyzed for AD and neuroinflammation markers.

Familial AD

- How does epileptic activity induced at early stages of AD affect the development of known AD pathological hallmarks?

To answer this, FAD ArcticAβ mice were injected with KA when they were 3 months old. Their AD pathology was immunohistochemically analyzed 6 months after the KA treatment.

Study II. Neurogenesis in ArcticAβ mice

The results of the former project showed that ArcticAβ mice are highly sensitive to KA. We wanted to explore possible mechanisms that underlie this hypersensitivity. Taking into account recent research, we hypothesized that adult neurogenesis could be a contributing factor.

- Is mutant hAPP overexpression or early increase in toxic intracellular Aβ species affecting adult neurogenesis in ArcticAβ mice?

We used BrdU labeling to estimate proliferation and survival rates of progenitor cells and viral labeling to visualize and analyse dendritic morphology of adult-born neurons.

Study III. Mechanisms of inhibitory synaptic strengthening

This project was done in collaboration with Derek Bowie's lab at McGill University in Montreal, Canada. It is focused on basic physiological mechanisms of synaptic strengthening, which could potentially be implicated in the changes in excitability in disease conditions Specifically, here, we are further investigating recently described mROS-induced GABAergic synaptic plasticity in a subpopulation of cerebellar interneurons (Accardi et al., 2014, Accardi et al., 2015).

- Can repeated increased input activity trigger un-silencing of a population of $GABA_A$Rs in the stellate interneurons?
- Is this type of synaptic strengthening mROS-dependent?
- Can this type of synaptic strengthening be induced by insulin?

To address these questions we performed whole cell patch clamp recordings of stellate interneurons. To detect strengthening of GABAergic synapses, we used extracellular minimal stimulation of input fibers to the recorded neuron. To un-silence $GABA_A$Rs we used repeated high frequency stimulation or bath application of insulin.

III. RESULTS

CHAPTER I: EFFECTS OF INDUCED SEIZURES ON ALZHEIMER DISEASE-LIKE PATHOLOGY IN SPORADIC AND FAMILIAL AD MOUSE MODELS

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In preparation for publication

Author's contribution

All the experimental procedures were carried out by MZ under the supervision of IK and JMF. MZ and JMF wrote the manuscript. Authors have no conflict of interest to declare.

Abstract

The vast majority of Alzheimer's disease (AD) cases are sporadic and only about one percent are familial. Since non-transgenic mice do not develop AD, it has been difficult to establish a mouse model of sporadic AD. Recently it was shown that prenatal inflammation induced by injecting pregnant dams with the viral mimic PolyI:C triggers long-term elevation of several cytokines and chemokines, which, coupled with a second immune challenge in adulthood, triggers symptoms of sporadic AD-like neuropathology with age. This "double hit" approach allows to model features of sporadic AD in wildtype mice.

In the recent years it has become increasingly accepted that AD and epilepsy may overlap. Clinically and experimentally, abnormal epileptic activity can occur prior to the onset of AD plaque pathology. Therefore, research is needed to disentangle the pathophysiological mechanisms common to both syndromes.

The aim of this study was to determine whether acquired seizures exacerbate AD-like pathology in sporadic and familial AD mouse models. We induced spontaneous recurrent seizures by unilateral intrahippocampal injection of kainic acid (KA) in wild-type sporadic "double hit" and transgenic ArcticAβ mouse models.

The results of experiments with sporadic AD mice were negative; however, we did not observe the expected effects of PolyI:C treatment on brain microglia, preventing a definitive conclusion. On the other hand, ArcticAβ mice had strongly increased sensitivity to KA. Transgenic animals that aged till they were 9 months-old exhibited a mild KA lesion with degeneration and neuroinflammation exclusively present in the CA1 region. Surprisingly, aged KA-injected ArcticAβ mice were devoid of Aβ plaques in the ipsilateral hippocampus and had a reduced plaque load in the contralateral hippocampus when compared to wild-type littermates. This finding suggests that local changes induced at pre-plaque stage by KA, such as neuroinflammmation or neurodegeneration, or possibly chronic seizure activity, prevent or at least delay plaque formation.

Introduction

Alzheimer's disease (AD) is one of the most prevalent age-related dementias. It is characterized by gradual loss of cognitive abilities, severe neurodegeneration and is associated with the formation of Aβ plaques and neurofibrillary tangles (NTFs) enriched with hyperphosphorylated tau.

For several decades evidence has been accumulating suggesting that AD and epilepsy might share common pathophysiological mechanisms. For instance, patients suffering from AD have 6 to 10 fold higher risk to develop seizures and epilepsy compared to healthy individuals of the same age (Sjogren et al., 1952, Hauser et al., 1986, Hesdorffer et al., 1996a, Amatniek et al., 2006, Scarmeas et al., 2009). Epileptic activity in some cases was shown to occur at the early "pre-degenerative" disease stages in AD patients (Hesdorffer et al., 1996a, Mendez and Lim, 2003, Amatniek et al., 2006, Scarmeas et al., 2009). On the other hand, epileptic patients have increased incidence of senile plaques (Mackenzie and Miller, 1994, Sheng et al., 1994, Gouras et al., 1997). Additional evidence came from AD mouse models, some of which have spontaneous seizures and neuropathological alterations reminiscent of those found in temporal lobe epilepsy (TLE) (Steinbach et al., 1998, Westmark et al., 2008, Palop and Mucke, 2010). TLE is a common type of epilepsy, which is characterized by severe neurodegeneration in hippocampal regions CA1 and hilus accompanied by reactive gliosis. Neuroinflammation (for instance, microglial activation, astrogliosis, elevation of inflammatory mediators) is a common feature in both AD and TLE.

The majority of AD cases are sporadic and fewer than 1% are familial; however, the mouse models used in AD research are mostly transgenic lines overexpressing familial AD gene products. To determine the causes of sporadic AD, different mouse models would be highly valuable. Our lab previously showed that wild-type (WT) mice prenatally exposed to an immune challenge induced by a viral-mimic PolyI:C show long-term elevation of several immune mediators and a predisposition to develop AD-like pathology during aging. A second exposure of these mice to PolyI:C (or second hit) in adulthood leads to the formation of senile plaques, reminiscent of those in humans, and causes cognitive deficits (Krstic et al., 2012). This mouse model is potentially a good candidate to study the development of sporadic AD.

The main goal of this project was to contribute to the understanding of the role of epileptic activity in the development of AD-like pathology by inducing chronic recurrent seizures in mice

predisposed to this disease. We used intrahippocampal kainic acid (KA) injection - a model widely used to mimic TLE, combined with pre- and postnatal exposure to PolyI:C. Typical KA lesion is characterized by neurodegeneration in the hippocampal region CA1, loss of interneurons, dentate gyrus granule cell dispersion, severe local microgliosis and astrogliosis and establishment of spontaneous recurrent seizures (SRS) after a two week long latent period.

The first part of the project focuses on the sporadic AD mouse model. Firstly, we wanted to determine whether in AD-susceptible brain, the induction of seizures could trigger AD pathology reminiscent of the one induced by the second PolyI:C hit (Group 1. prenatally PolyI:C treated mice). Next, we were interested whether epileptic activity together with an immune challenge in adulthood could induce AD-like pathology during ageing (Group 2. postnatally PolyI:C treated mice).

The second part of the project focused on a familial AD mouse model, namely ArcticAβ mice. Those mice express human APP with two familial AD mutations, leading to an increase in toxic Aβ species and, starting at 7 months of age, Aβ plaques formation (Knobloch et al., 2007). With induction of seizures at pre-plaque stages in ArcticAβ mice we wanted to see whether this exacerbates the AD-like pathology that develops with ageing.

Materials and Methods

Animals

All experiments performed in this study were approved by the local authorities of the Cantonal Veterinary Office of Zurich and were carried out in accordance with Swiss Law on animal experimentation.

Mice used in the experiments:

- wild-type C57Bl6/JOla mouse strain (Harlan Laboratories, Horst, the Netherlands)
- transgenic ArcticAβ (Knobloch et al., 2007), and their age-matched wild-type littermates (bred at the Laboratory Animal Sciences Center of the University of Zürich)

All animals were housed in an in-house standard hygiene facility with access to food and water *ad libitum* under 12-hour light/dark cycle at the Institute of Pharmacology and Toxicology, University of Zurich, Switzerland. Both males and females were used in this study.

Polyriboinosinic-Polyribocytidilic Acid (PolyI:C) injections

Pregnant C57Bl/6JOla mouse strain dams were shipped on gestational day (GD) 14 and at GD17 intravenously (i.v.) injected with PolyI:C (5mg/kg) potassium salt (P9582, 50 mg; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) dissolved in 0.9% sterile NaCl. Injection volume of PolyI:C or saline was 5mL/kg body weight. During the injection procedure, the animals were restrained in acrylic mouse restrainer (PlasLabs, Inc. #561-RC). After the injection, dams were returned to their home cage. A group of offsprings for a part of the project was i.v. injected with PolyI:C or NaCl at the age of 3 months.

Intrahippocampal kainic acid (KA) injection

Two months old mice were anaesthetized with inhalation of 2.5-3% isoflurane (Baxter) in oxygen and received stereotactic injection of 70nL KA (5mM in NaCl; Tocris biosciences) or an equivalent volume of NaCl into the right dorsal hippocampus (stereotactic coordinates relative to Bregma: anteroposterior (AP) - 1.8mm, mediolateral (ML) - 1.6mm, dorsoventral (DV) - 1.9mm). Prior and after the surgery mice were intraperitoneally (i.p.) injected with 1mg/kg buprenorphine (Temgesic, Reckitt Benckiser AG, Switzerland).

Tissue preparation for immunohistochemistry

Brain tissue of 9 or 15 months old animals was collected and treated according to the protocol described by Notter et al. (2014). Mice received an intraperitoneal injection of an anesthetic (Nembutal; 50mg/kg) and intracardially perfused with 15-20 mL at a flow rate of 10-15 mL/min by ice-cold, oxygenated aCSF (in mM: NaCl 125, KCl 2.5, $CaCl_2$ 3.7, $MgCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, glucose 25). The brain was removed from the cranium and a block of brain tissue containing dorsal hippocampus was extracted. The block was immediately immersion fixed for 3 hours in ice-cold fixative (4% paraformaldehyde in 0.15 M sodium phosphate buffer), pH 7.4. The block was rinsed with phosphate buffer saline (PBS) prior the cryoprotection in 30% sucrose in PBS overnight at 4 °C.

Fixed tissue was cut into 40µm thick coronal sections using sliding microtome and collected in ice-cold PBS. For long term storage at -20 °C, sections were placed to a cryoprotectant solution (50 mM sodium phosphate buffer, pH 7.4, containing 15% glucose and 30% ethylene glycol; Sigma-Aldrich).

Immunohistochemistry

Sections were rinsed in Tris buffer (50mM Tris, 150mM NaCl, 0.05% Triton X100, pH 7.4) 3x10 minutes and incubated with primary antibody diluted in Tris buffer with 2% normal goat serum (NGS) and Triton X-100 overnight at 4°C under continuous agitation. Sections were rinsed 3x10 minutes in tris buffer and incubated with the secondary antibody solution (2% NGS, Tris buffer) for 30 minutes at room temperature. Biotinylated secondary antibodies for immunoperoxidase staining (Jackson ImmunoResearch Laboratories) were diluted 1:300.

Target	**Description**	**Dilution**	**Distributor**	**Procedure**
CD68	rat	1:2000	AbD Serotec	IF,
APP (C-terminal)	rabbit	1:60000	Immunogens	IF
$A\beta_{1\text{-}40/42}$	rabbit	1:2000	Millipore/Chemicon	IF
5-HT	rabbit	1:5000	Immunostar	IF
TH	rabbit	1:3000	Chemicon	IF

Table 1. List of primary antibodies. IF – immunofluorescence.

After washing 3x10 in Tris buffer, sections were incubated in avidin-peroxidase-complex (Vectostain Elite Kit, Vector Labs) for 60 minutes at room temperature. After 3x10 minutes rinse

in Tris buffer sections were mounted on gelatinized glass slides, air-dried overnight and dehydrated the next day through multiple ethanol and xelene steps and coversliped with.

Nissl staining

Air dried coverslips with mounted sections were submerged in the following solutions: 5 min in dH_2O, 5 minutes in filtered Cresyl violet solution ($C_{18}H_{15}N_3O_3$; M 321.34, Fluka BioChemika, Cat.no. 10510-54-0), 30 sec in dH_2O and cleared in 96% ethanol containing 0.5% acetic acid until the desired coloration was obtained, 5 minutes in isopropanol, 5 minutes in isopropanol:xylene (1:2) and 4 times dehydrated in xelene for 2 minutes. Afterwards slides were coversliped with resinous (Eukitt; Sigma-Aldrich) mounting medium.

Image acquisition

For visual assessment of Nissl stainings, 3-4 sections per animal were examined using an Axioscop 2 microscope (Carl Zeiss) with bright field illumination with 10x, 20x or 40x objectives. Images were acquired with color digital camera (AxioCam MRc5) and a AxioVision 4.5 software (Zeiss).

Densitometry

Intensity of immunoperoxidase staining was measured with densitometry analysis using the MCID software (MCID Elite 6.0, Interfocus Imaging Ltd., Cambridge, UK). Images of single sections were digitized with a precision illuminator (Northern light Model B95, Imaging Research Inc., Brock University, St. Catherines, Canada) and CoolSnap camera (Photometrics, Tuscon, AZ, USA) With Micro-Nikkor (55 mm+12 mm) lens (Nikon Corp.) obtained grey values were calibrated (Kodak step tablet no 310ST607). The intensity of different regions of interest was measured. The individual staining intensity was normalized to the intensity of the whole section. 3-4 sections per animal were analyzed.

Stereology

Images of individual sections were obtained using Axioplan 2 bright-field microscope (Carl Zeiss AG, Feldbach, Switzerland) with 20x lens (air, NA0.75) and a digital camera (MicroFire, Optronics AG, Goldbach, Switzerland). The volume of the hippocampus and cerebral cortex and number of plaques were estimated with Mercator software (Mercator Pro rev. 7.8.2.; Explora Nova, La Rochelle, France).

Statistical analysis

One-way or two-way ANOVA with Bonferoni post-hoc test was performed for multiple comparison groups. To compare two groups an unpaired two-tailed t-test was used. Statistical analysis was performed in Microsoft Excel and Prism software (GraphPad v.6).

Results

Part 1. Effects of acquired seizures on AD-like pathology in sporadic AD mouse model

To determine whether in AD- susceptible brain, the induction of seizures could trigger AD-like pathology reminiscent of the one induced by the second PolyI:C hit (Krstic et al., 2012), the offspring of WT mice intravenously injected with PolyI:C at gestational day 17 (GD17) were taken into experiments at the age of 3 months and were injected intrahippocampally with KA. At the age of 9 or 15 months their brain tissue was collected and immunohistochemically analyzed for AD and neuroinflammation markers.

The extent of the KA-induced lesion and cellular reorganization of the dorsal hippocampus was determined by visual assessment of Nissl-stained sections at both time-points, and compared with control mice treated with saline instead of PolyI:C (Fig. 1). No phenotypic differences were observed at either 9 or 15 months post-KA.

CD68 was used as a marker to assess the extent of microglial activation. Densitometric analysis showed that in both prenatally PolyI:C treated and NaCl treated controls microglial activation was elevated and sustained after KA treatment in the ipsilateral hippocampus. There was no difference in the extent of microglial activation in both groups of animals at either 9 or 15 months of age (Figure 2). Tissue was also stained with an antibody recognizing a C terminal epitope of APP, labeling all APP isoforms. Densitometric analysis showed that APP-immunoreactivity was slightly elevated in the granule cells of the dentate gyrus to the same extent in prenatally PolyI:C treated and NaCl controls in both 9 and 15 months age groups (Figure 3). Interestingly, APP was increased contralateraly in CA1 stratum pyramidale in prenatally PolyI:C treated mice in the 15 months group (1.31±0.04, n=6; 1,18±0.02, n=6; mean ± s.e.m., P=0.013) (Figure 3, d.).

It is also important to note that generally extensive microglial activation was present at the ipsilateral hippocampus up to 12 months after KA injection.

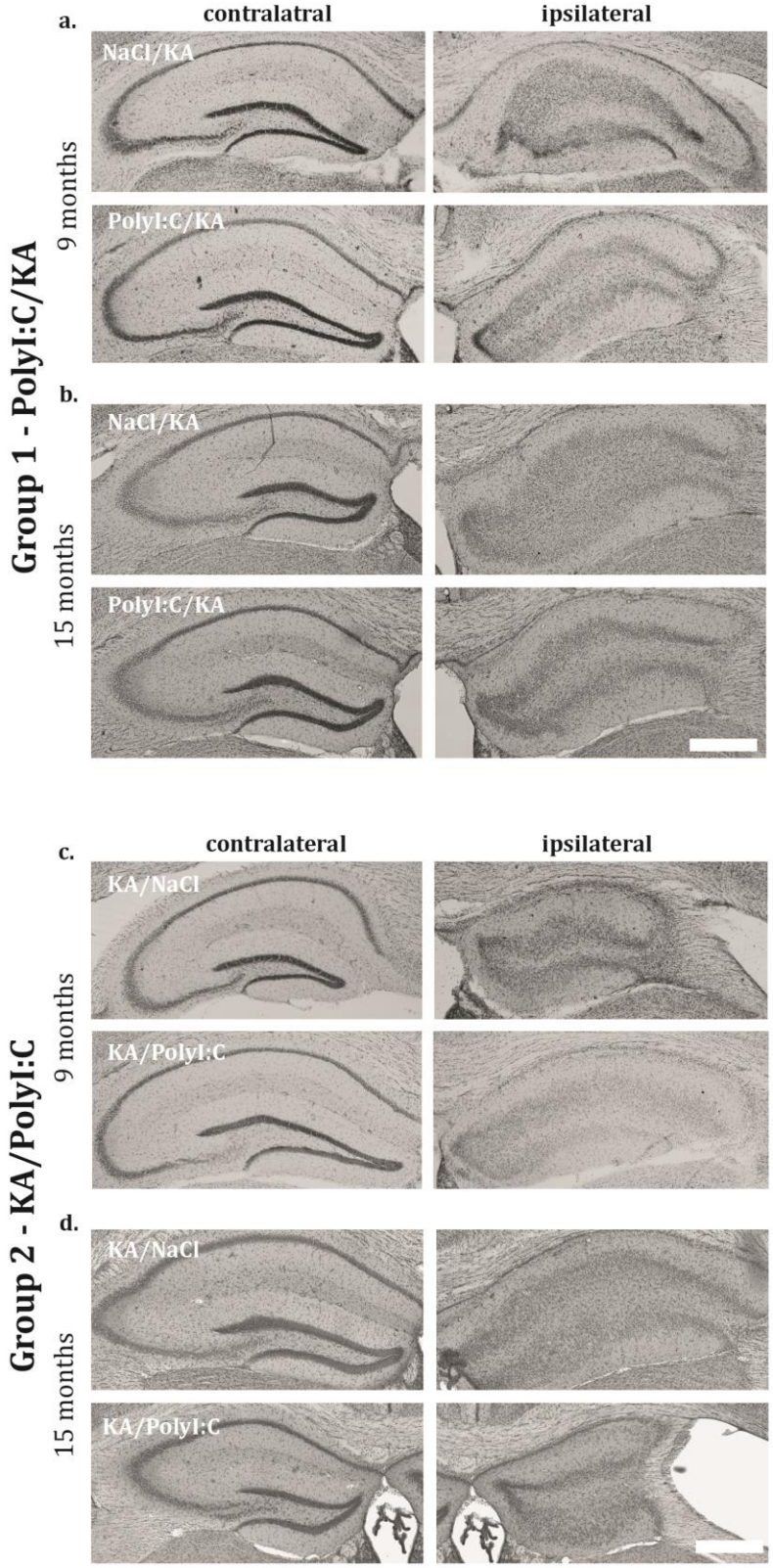

Figure 1. Nissl stainings of ipsilateral and contralateral dorsal hippocampus of mice unilaterally injected with KA. KA lesions in prenatally (**a,b**) and postnatally (**c,d**) Poly:C treated mice sacrificed at 9 (**a,c**) and 15 (**b,d**) months. Scale bar – 250µm.

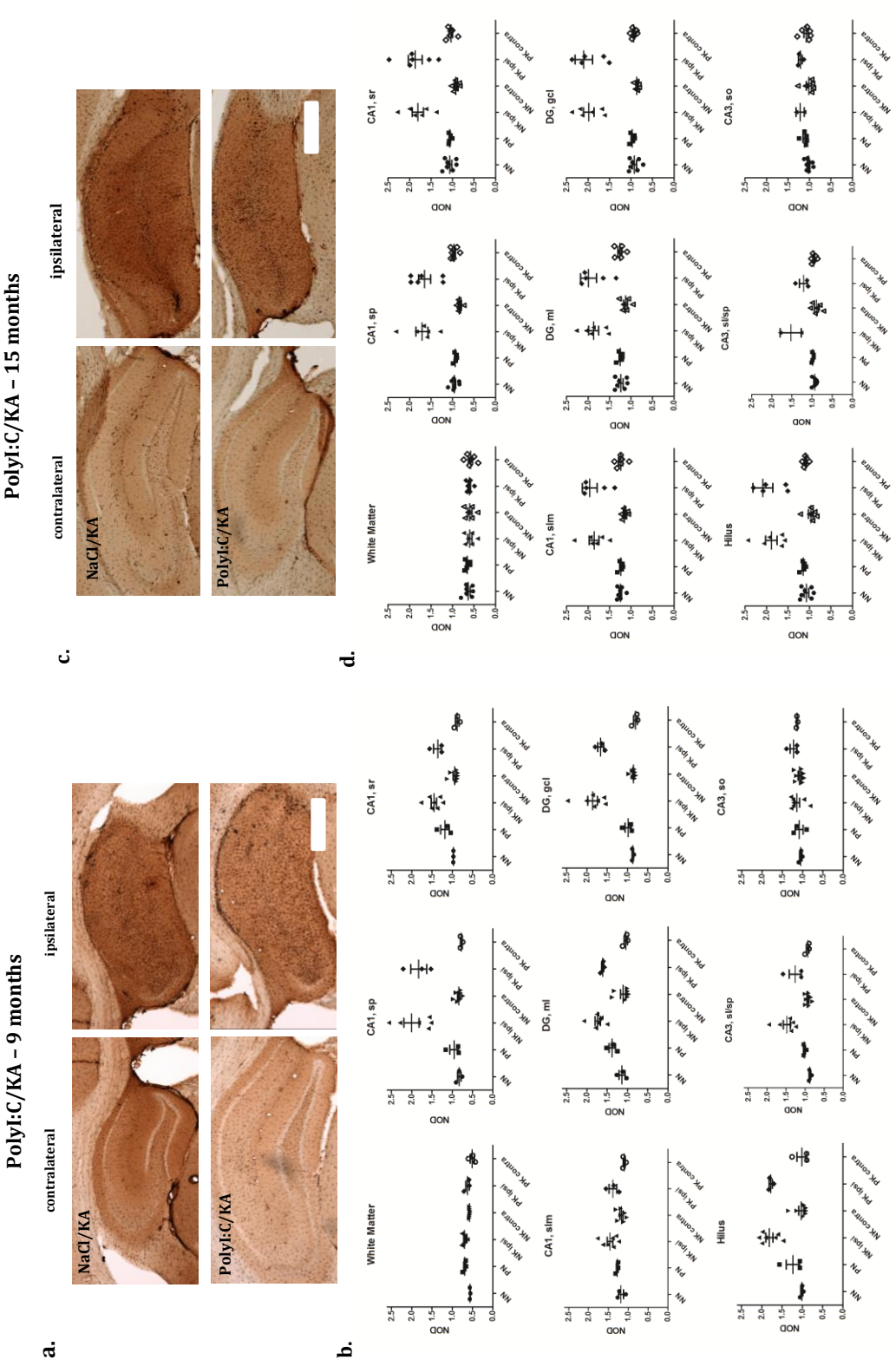

Figure 2. Effects of acquired seizures on microglial activation in prenatally PolyI:C challenged mice. Immunoperoxidase staining for CD68 microglial marker in dorsal hippocampus (**a,c**) of 9 (**a**) and 15 (**c**) months old mice injected with kainate. Ipsilateral hippocampi show extensive presence of activated microglia typical for the KA injected brain. (**b,d**) Densitometric analysis of immunoperoxidase CD68 staining throughout the layers of dorsal hippocampus of 9 (**b**) and 15 (**d.f**) months old KA injected animals. Scale bar – 300 μm.

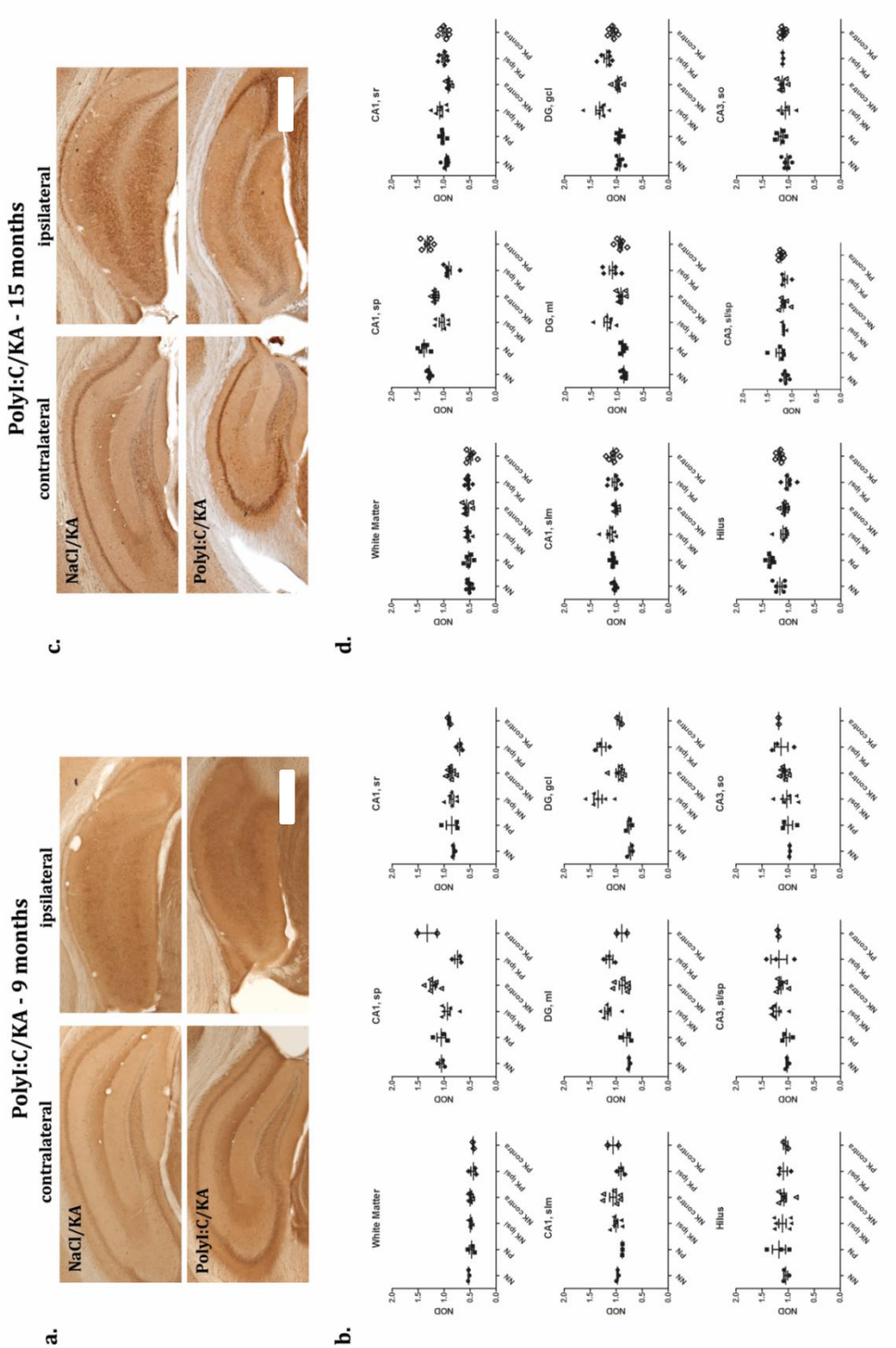

Figure 3. Effects of acquired seizures on APP pathology in postnatally PolyI:C challenged mice. Examples of immunoperoxidase staining for C terminal APP in dorsal hippocampus **(a,c)** of 9 **(a)** and 15 **(c)** months old mice injected with kainate. **(b,d)** Densitometric analysis of immunoperoxidase APP C-term staining throughout the layers of dorsal hippocampus of 9 **(b)** and 15 **(d.f)** months old KA injected animals. Scale abr – 300 μm.

Effects of immune challenge on AD pathology in epileptic mice

To check if epileptic activity together with an immune challenge in the adulthood could induce AD-like pathology, WT mice were injected with KA at 3 months of age and 1 month later they were intravenously injected with PolyI:C. Mice were sacrificed at 9 and 15 months of age and their brain tissue was collected and immunohistochemically analyzed.

Overall elevation of CD68 microglial marker was observed in all the hippocampal areas in all age groups of mice, but was not affected by PolyI:C treatment in adulthood (Figure 4). APP was elevated in response to KA injection, but to similar levels in all the treatment groups at both 9 and 15 months (Figure 5). We did not observe expected immune challenge induced increase in APP and CD68 levels in 9 or 15 months group. Such effects of PolyI:C challenge would have been fundamental in our experiments and we did not go on further with the analysis of this tissue.

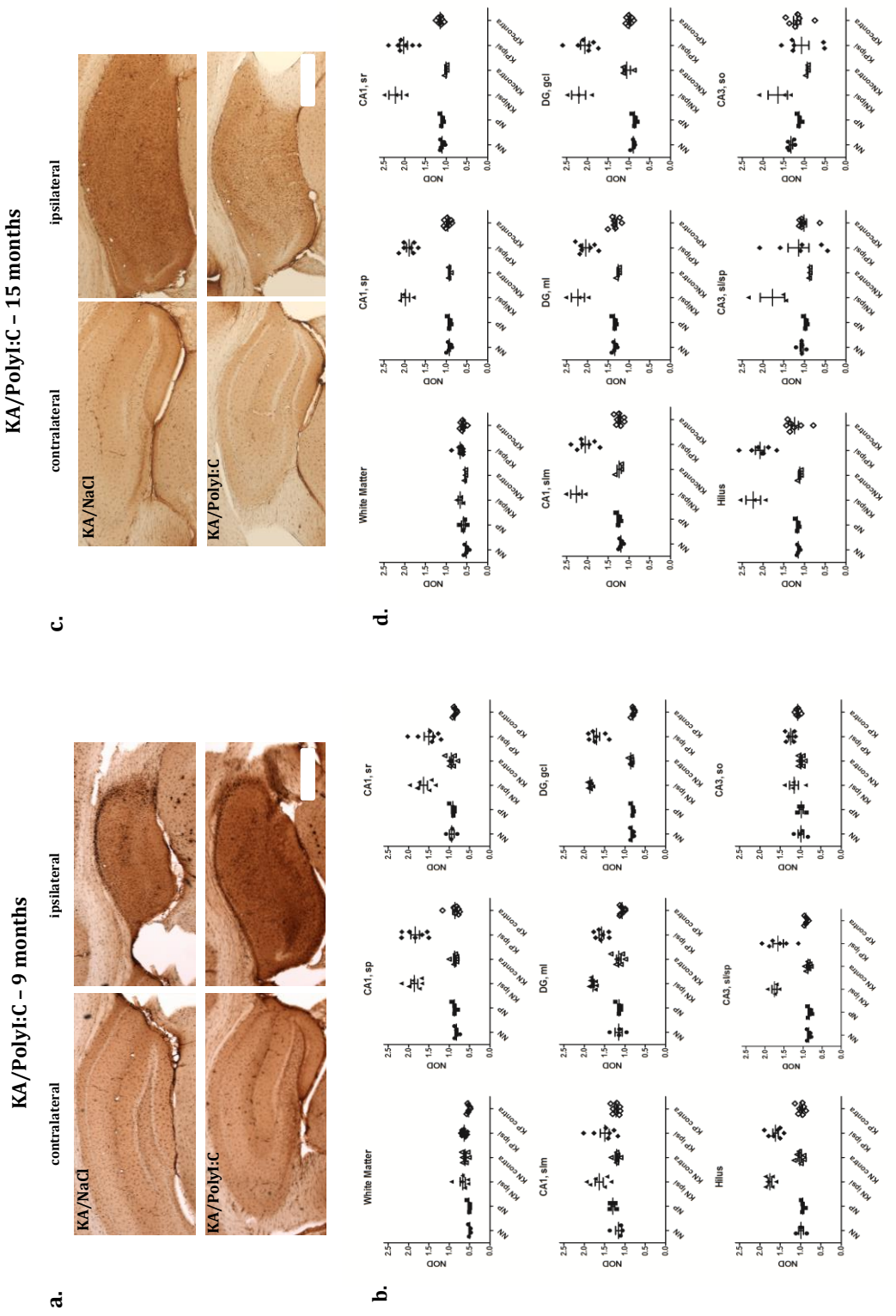

Figure 4. Effects of immune challenge on microglial activation in mice with chronic epileptic activity. Examples of immunoperoxidase staining for C terminal APP in dorsal hippocampus **(a,c)** of 9 **(a)** and 15 **(c)** months old mice injected with kainate. **(b,d).** Scale bar – 300 μm.

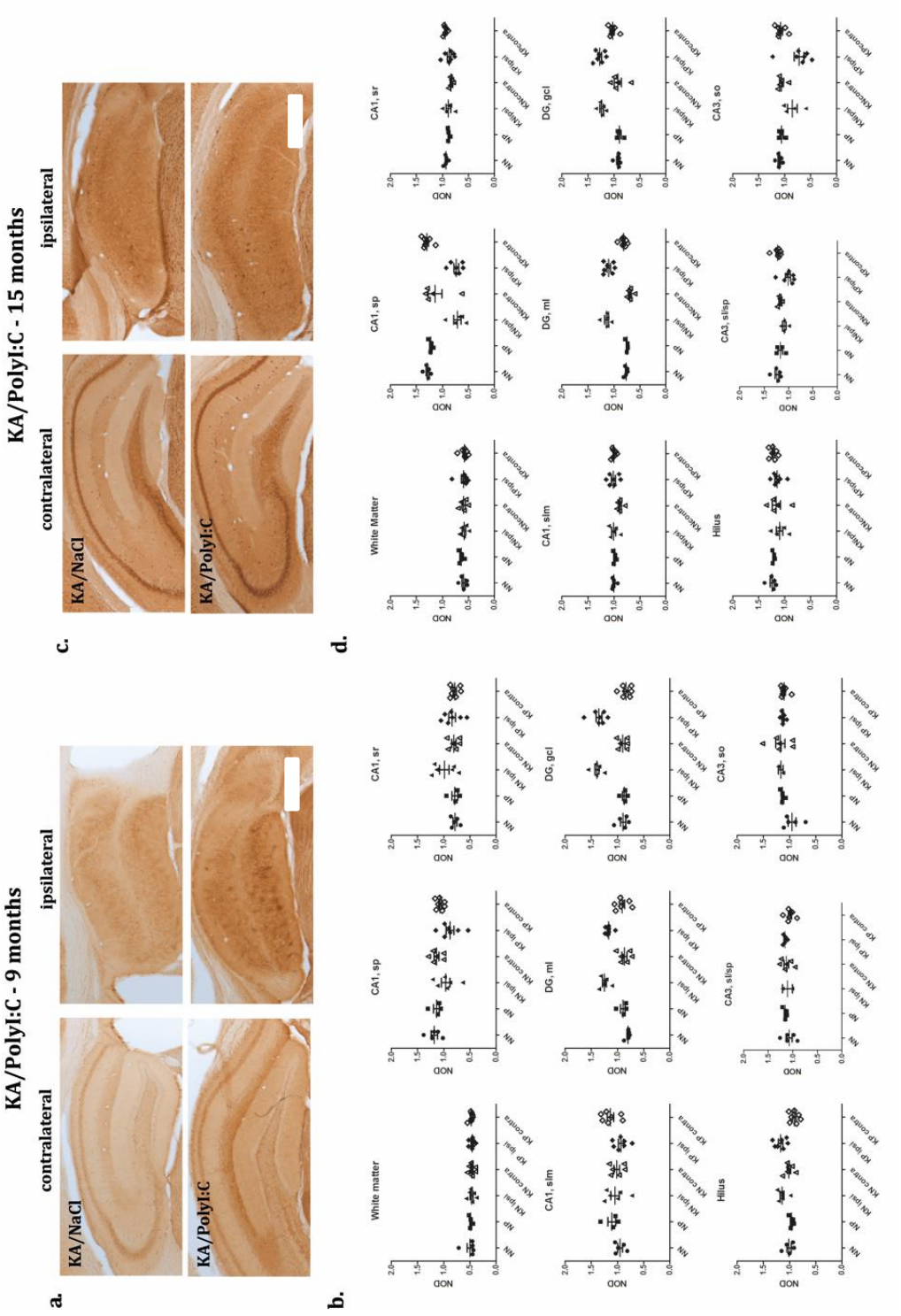

Figure 5. Consequences of immune challenge on APP expression in mice with chronic epileptic activity. Examples of immunoperoxidase staining for C terminal APP in dorsal hippocampus **(a,c)** of 9 **(a)** and 15 **(c)** months old mice injected with kainite, prior PolyI:C challenge **(b,d)**. Densitometric analysis of immunoperoxidase APP C-term staining throughout the layers of dorsal hippocampus of 9 **(b)** and 15 **(d.f)** months old KA injected animals. Scale bar – 300 μm.

Part 2. Effects of acquired seizures on AD-pathology in familial AD mouse model

To understand how recurrent seizures, induced during the pre-plaque stages affect the development of AD-like pathology we combined AD and TLE mouse models. To induce life-long SRS, a group of 3 months old ArcticAβ mice and control littermates were intrahippocampally injected with KA. In a first cohort of mutant mice, only 40% survived the KA-injection due to strong status epilepticus. Therefore, upon recovery from anesthesia, the mice received one intraperitoneal injection of diazepam (5mg/kg) to prevent mortality during the KA-induced status epilepticus. Brain tissue was collected 9 months later and was processed for immunohistochemistry. In ArcticAβ mice, diazepam fully prevented the immediate (first 24h) post-KA mortality; however, in the course of the first 40 days after the KA injection, only 27% of injected transgenic mice survived. All the wild-type littermates recovered normally after the KA injection and aged till 9 months.

"Mild" phenotype of KA lesions in ArcticAβ mice

As a consequence of this mortality, ArcticAβ mice that survived during the experiment had a mild KA lesion phenotype, as seen in Nissl-stained sections, represented by degeneration of pyramidal neurons in the hippocampal CA1 region limited to the lesion site (Figure 6). Wild-type animals had the "usual" lesion phenotype, characterized by CA1 degeneration, granule cells dispersion and extensive microglial activation. Therefore, the survivors probably received a mild initial lesion and might not be truly representative of the whole cohort.

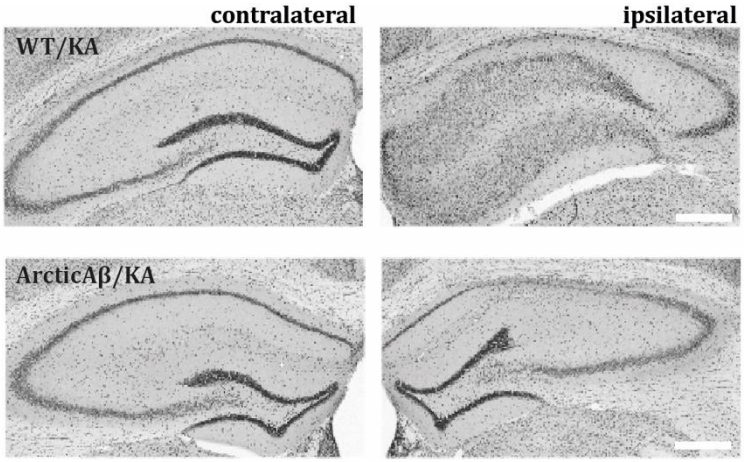

Figure 6. KA lesions in ArcticAβ mice. Nissl staining of dorsal hippocampus typical KA lesions of 9 months old ArcticAβ mice and WT controls. KA lesions in ArcticAβ have mild phenotype with CA1 pyramidal cell layer degeneration and no granule cell layer dispersion. Scale bar – 250 μm.

Decrease of plaque load in KA injected ArcticAβ mice

Stereological quantification of Aβ positive plaques revealed their significantly smaller number of in both ipsilateral and contralateral (to the KA lesion) hippocampi of ArcticAβ mice in comparison to NaCl injected transgenic controls. Hippocampus of KA injected animals had significantly lower number of plaques in the ipsilateral side if compared to the contralateral side.

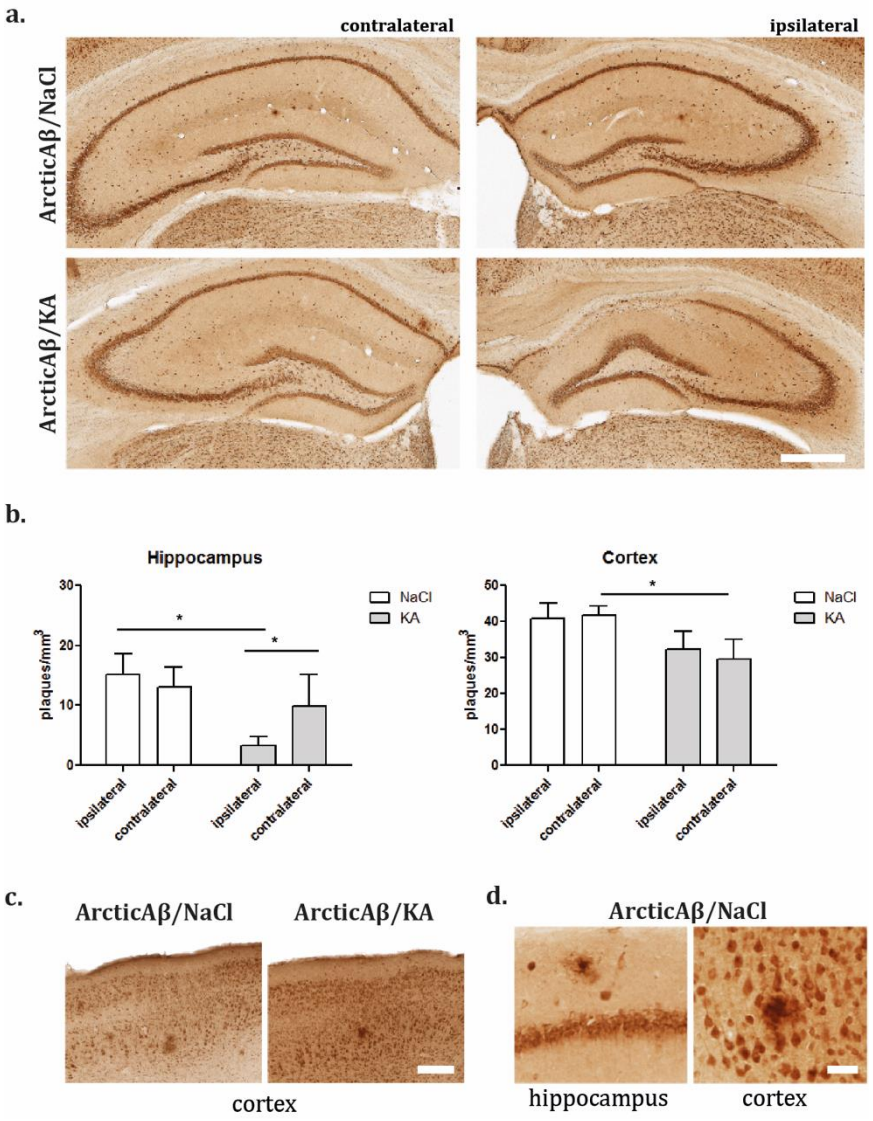

Figure 7. Aβ plaque pathology in KA injected ArcticAβ mice. (a) Immunoperoxidase staining of dorsal hippocamopus of ArcticAβ mice injected with either NaCl or KA. Scale bar-250 μm. **(b)** Stereological quatification of the number of plaques in dorsal hippocampous and cortex of ArcticAβ NaCl and KA injected mice. **(c)** Example images of Aβ plaques in the cortex of Arctic mice injected with KA or NaCl. Scale bar -150μm **(d)** Plaque pathology in the hippocampus (ipsilateral) and cortex of ArcticAβ injected with NaCl. Scale bar - 50 μm.

A trend towards a reduced number of plaques was observed in the cortex of ArcticAβ KA injected group. But the plaque number did not differ at the ipsilateral and contralateral sides of

the cortex within the treatment group (Figure 7).

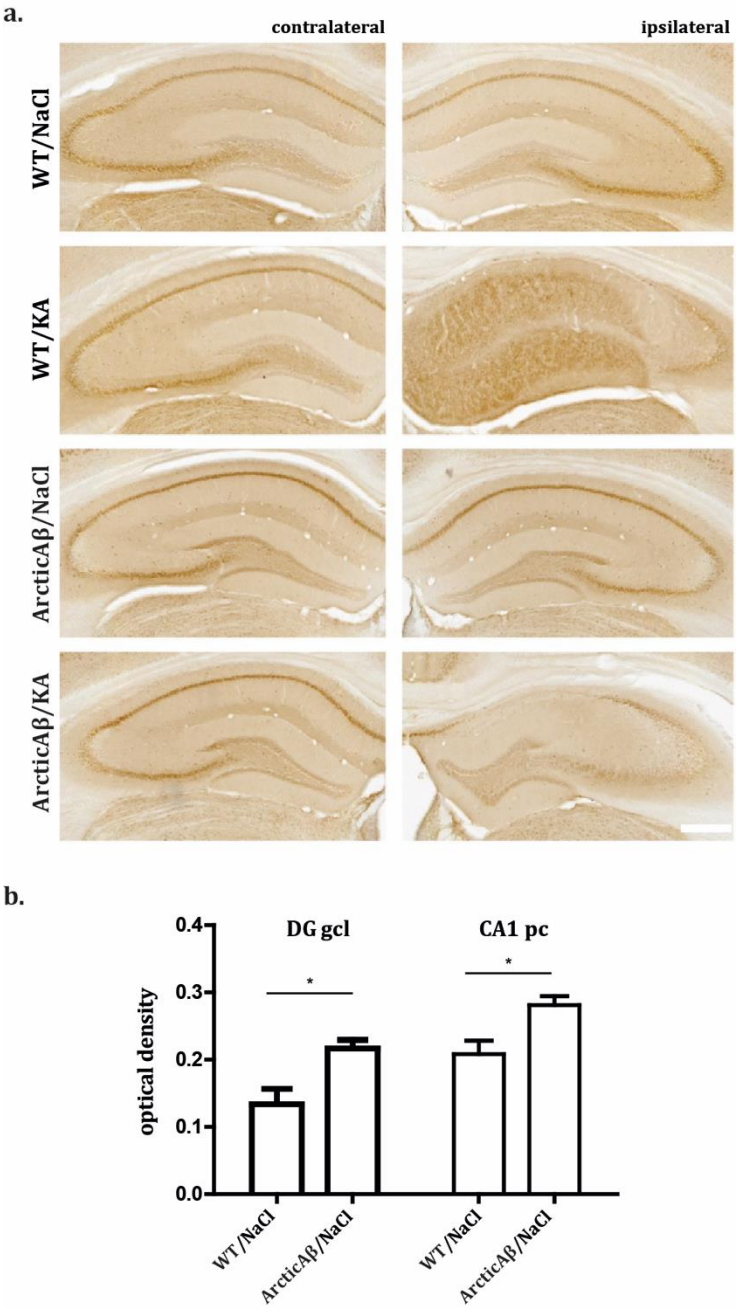

Figure 8. APP expression in KA injected ArcticAβ mice. (a.) Immunoperoxidase staining for C-terminal APP of ArcticAβ and WT mice injected with KA or NaCl. Scale bar – 250 μm **(b.)** Desitometric analysis of the staining showing an increase in APP optical density in DG granule cell layer and CA1 pyramidal cell layer.

Effects of KA injection on APP expression on ArcticAβ mice

As transgenic animals overexpress APP, densitometric analysis of immunostaining against C-terminal APP showed an increase in the hippocampal granule cell layer and the CA1 pyramidal cell layer in ArcticAβ animals in comparison to WT (Figure 8). The analysis of the staining showed no differences in changes of APP levels after KA between the between NaCl and KA injected ArcticAβ mice.

Microgliosis in KA injected ArcticAβ mice

Immunoperoxidase stainings for microglial marker CD68, showed extensive microglial activation in the entire ipsilateral hippocampus was present even 6 months after the KA injection in WT mice. In ArcticAβ mice activated microglia was only present in degenerating CA1 region, but otherwise was not different from NaCl-injected controls (Figure 9).

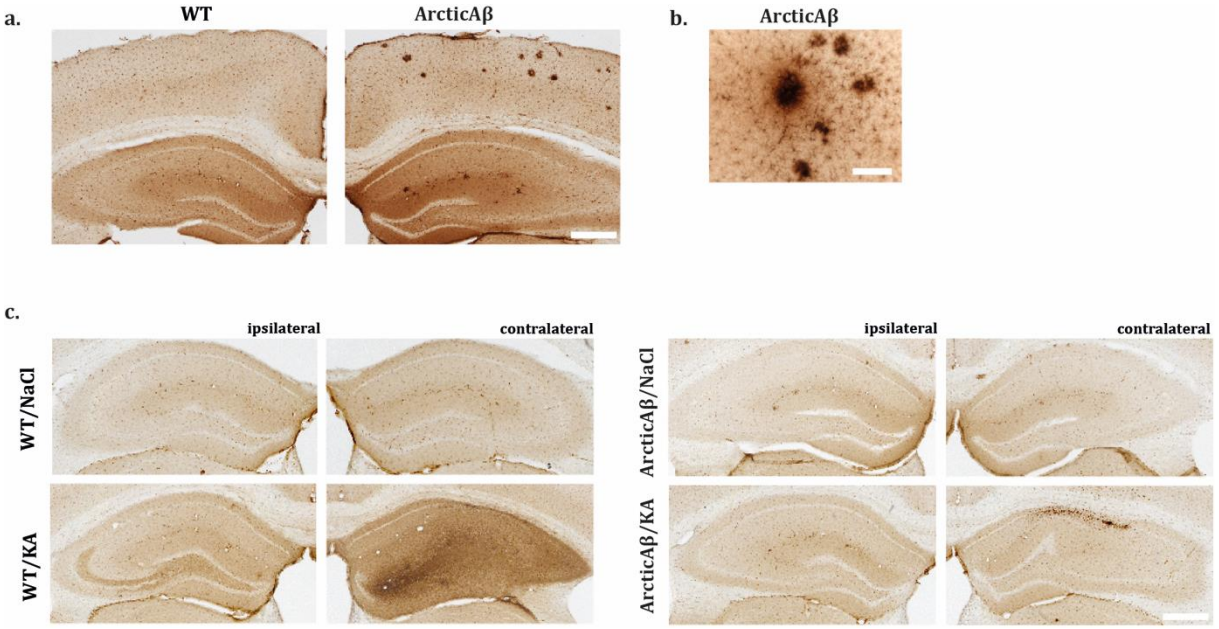

Figure 9. Microglial activation in response to KA injection in ArcticAβ mice. (a.) Immunoperoxidase staining for CD68 in WT and ArcticAβ mice. ArcticAβ mice showing accumulations of microglia around Aβ plaques in the cortex and hippocampus (right). Scale bar – 250 μm **(b.).** Examples of microglial accumulations in the cortex of ArcticAβ mice. Scale bar – 50 μm **(c.)** Dorsal hippocampus of WT (left) and ArcticAβ (right) mice injected with KA or NaCl. Scale bar – 250 μm.

No changes in subcortical innervation in dorsal hippocampus in response to KA treatment in ArcticAβ mice with ageing

Del Tredici and Braak (2013) suggested that Aβ plaques form in the projection zones of noradrenergic and serotonergic fibers. We hypothesized that the absence of Aβ plaques could be

related to the changes of subcortical innervation of hippocampus, as a result of KA treatment. Visual assessment of sections processed for tyrosine hydroxylase and 5-HT immunoreactivity revealed that those inputs are not affected at the KA lesion sites in either genotype (Figure 10).

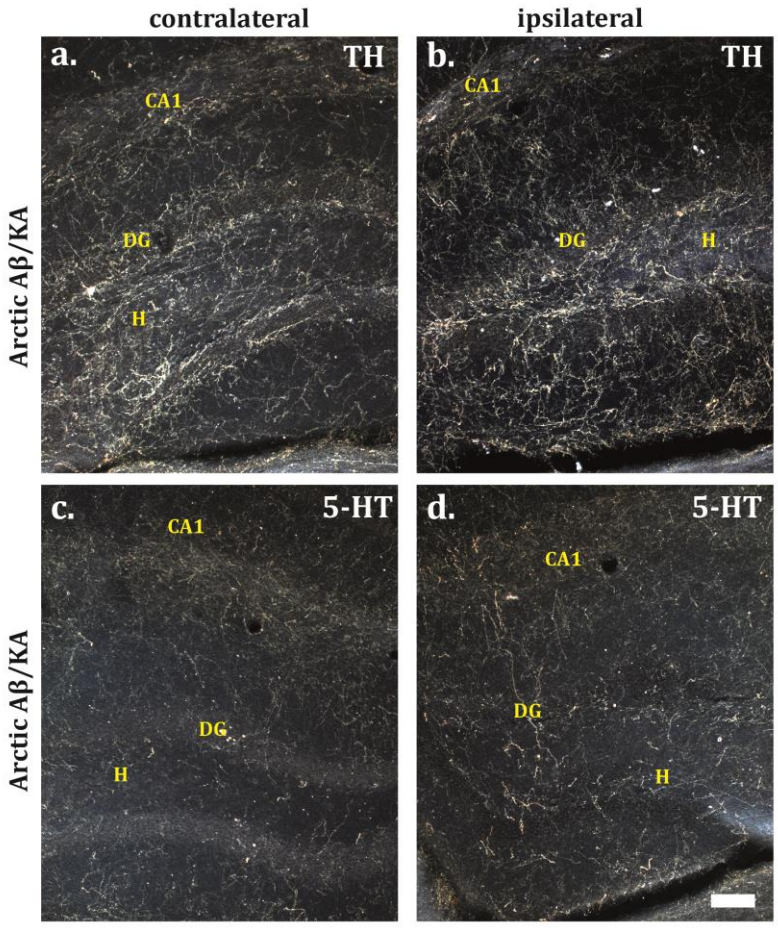

Figure 10. Noradrenergic and serotonergic fibers in the KA lesioned hippocampi of ArcticAβ mice. (a,b) Immunoperoxidase staining for Tyrosine hydroxylase (TH) and **(c,d)** 5-Hydroxytryptamin (5-HT) of the ipsilateral (**b,d**) and contralateral (**a,c**) dorsal hippocampus of ArcticAβ mice injected with KA.CA1-cornu ammonis; DG – dentate gyrus; H- hilus. Scale bar – 250 μm.

Discussion

In this project, we were looking at the effects of epileptic activity on the development of AD pathology in sporadic "double hit" and familial ArcticAβ mouse models. Inducing seizures in prenatally PolyI:C challenged mice, as well as an attempt to exacerbate the effect of seizures by a PolyI:C hit in adult mice did not lead to the development of AD-like pathology with ageing. In fact, those experiments remain inconclusive because we did not observe the expected age-related effects of PolyI:C challenges in non-transgenic mice, which was central to a concept of this sporadic AD mouse model.

ArcticAβ were very sensitive to KA injection and the experimental group had a drastic seizure-related mortality rate. Surprisingly, KA injected animals at 9 months of age had no plaques in the ipsilateral hippocampus and a tendency to decreased plaque load in other brain areas.

Sporadic AD

The results of the present study show no effects of induced seizures on the development of AD pathology in prenatally immune-challenged mice. Likewise, neuroinflammation and SRS caused by KA injection at adult stages could not mimic the effects of the "second hit" of the "double hit" PolyI:C mouse model, except for a slight elevation of APP at the contralateral to the injection site in PolyI:C prenatally treated mice. SRS and neuroinflammation in TLE KA model are localized to the ipsilateral hippocampus. It would be interesting to combine both prenatal and adult PolyI:C hits with KA injection to see whether the latter would aggravate AD pathology even more.

Immune challenge that followed KA injection did not lead to AD pathology in WT mice with ageing. This result might be interpreted as evidence that a prenatal immune challenge impacting on immune system development might be crucial for triggering a later risk to develop AD-like pathology.

Because we could not observe any effect of PolyI:C after either prenatal exposure or postnatal injection, the present experiments remain unconclusive. Several reasons might explain a possible lack of effect of PolyI:C. For instance, the original PolyI:C experiment was performed in C57Bl/6JRcc mouse strain (Krstic et al., 2012), but in our studies we used C57Bl/6JOla mouse strain. The latter strain has a spontaneous mutation affecting α-synuclein expression. α-synuclein

is a protein present in synaptic terminals throughout the brain and is an important player in the development of Parkinson's disease (Stefanis, 2012). Interestingly α-synuclein is also considered to play a role in peripheral immune system (Kim et al., 2004) and could potentially interfere with the response of the immune system to PolyI:C. Another impediment that might have interfered with the effects of PolyI:C could be handling of pregnant dams around the injection time. In the original "double hit" experiment pregnant females were injected "in house" without any additional transport (Krstic et al., 2012). In our case, they had to be transported from the animal provider at GD14 before being injected 3 days later. It is known that transport and acclimatization affects glucocorticoid levels in rodents (Landi et al., 1982, Tuli et al., 1995). Three days of acclimatization could be not enough to stabilize stress hormones levels and this could have interfered with the PolyI:C treatment.

Familial AD

To test whether induced seizures affect AD-like pathology in a transgenic AD mouse line, we mimicked TLE in ArcticAβ mice, that overexpress human APP carrying multiple mutations. In comparison to WT, transgenic animals had increased mortality after KA injection. Prevention of the initial post-surgical status epilepticus with diazepam delayed, but did not diminish the mortality of injected animals. Mice presumably died as a result of seizures within the next 2 months post KA injection. This unexpected observation suggests that ArcticAβ mice have increased seizure susceptibility and lower threshold to status epilepticus development already at pre-plaque stages. It was shown that at the age of 2 months, when the mice were injected with KA, in the absence of Aβ plaques, intracellular Aβ levels are already elevated, suggesting that increase in toxic Aβ species could potentially be epileptogenic. ArcticAβ mice have two FAD mutations, namely Swedish and Arctic. Recently, it was shown that mice carrying only Arctic mutation (ArcAPP mice) have mild epileptic phenotype in comparison to animals expressing APP with Swedish mutation, despite higher APP overexpression (Ziyatdinova et al., 2016). Together with ours, this finding underscores the role of increased β secretase activity, characteristic for Swedish APP mutation, in epileptogesis in ArcticAβ mice.

Mice that survived until 9 months of age after KA injection had a "mild" lesion phenotype, that could be mainly recognized by the degeneration of CA1 at the injection site in dorsal hippocampus. Although, the majority of WT mice had the typical lesion phenotype, with CA1 degeneration, granule cells dispersion and extensive microglial activation, a few also had a mild lesion phenotype, indicating that there is a certain degree of variability in KA injections. Mutant

mice with more severe lesions probably died from convulsive seizures during the first month after the injection. We assume that the severity of lesions is directly linked to the survival of ArcticAβ mice. "Mild" phenotype of KA lesions in 9 months old KA injected group implies neurodegeneration and microgliosis limited to the CA1 region of the dorsal hippocampus and it was shown that destruction of CA1 may be enough for the development of SRS (Arabadzisz et al., 2005, Antonucci et al., 2008).

Contrary to our expectations induction of seizures in ArcticAβ mice did not cause an increase in Aβ plaque pathology at 9 months. Rather, a tendency towards a reduced number of plaques was observed in the cerebral cortex of the ArcticAβ mice, but plaque density did not differ in the ipsilateral and contralateral sides of the cortex. In this transgenic mouse line plaques form late and rather slowly (Knobloch et al., 2007). Those survivors could be the outliers that would normally develop plaque pathology later. A tendency towards reduced plaque pathology in some of the injected ArcticAβ mice could in combination with a mild KA lesion contribute to the survival of those animals. However, this cannot explain the absence of plaques in the lesioned hippocampus. It was proposed (Del Tredici and Braak, 2013) that Aβ plaques form (more or less exclusively) in the projection zones of noradreneric and serotonergic fibers originating from locus coeruleus and upper raphe nuclei respectively. We hypothesized that if KA lesion causes functional and input isolation of ipsilateral hippocampus, this could prevent the Aβ deposition. From immunolabeling for TH and 5-HT it turned out that noradrenergic and serotonergic inputs are preserved in the ipsilateral and contralateral hippocampi of ArcticAβ and WT mice. This disproves our assumption and suggests that the absence of plaques in the ipsilateral hippocampus at 9 months is probably a result of local changes induced by KA. Long-lasting neuroinflammation triggered by KA at the pre-plaque stages could play a role in the absence of plaques in the ipsilateral hippocampus. Apart from microgliosis KA lesion-related neuroinflammation is characterized by brain infiltration of peripheral macrophages, an event that is in fact shown to be neuroprotective (Zattoni et al., 2011). The characteristic KA induced neuroinflammation in young ArcticAβ mice formation could prevent or delay plaque formation. The age onset of plaque pathology in ArticAβ mice is 7-9 months (Knobloch et al., 2007). Therefore, the group of surviving mice could represent a group of mice in which plaque pathology develops later. To check this looking at later timepoints would be beneficial.

Activated microglia, as visually estimated from the immunostaining for CD68, was observed in the ipsilateral hippocampus in ArticAβ mice, but limited only to the degenerating CA1

pyramidal cell layer, which was presumably the only area affected by KA treatment in the group of surviving mice. As in previous experiments, extensive microglial activation was a prominent feature of the ipsilateral KA injected hippocampus in the WT controls.

Analysis of immunostaining against C terminal APP showed an increase in optical density in ArcticAβ mice in comparison to WT controls. This is explained by the overexpression of the protein in transgenic mice. APP staining was not denser in the ipsilateral or contralateral hippocampus of the transgenics in comparison to the WT controls. To make a solid conclusion about the APP expression, limitations of immunolabeling have to be taken into account. To confirm this result, it would be interesting to use Western blotting to estimate the levels of APP expression.

Increased sensitivity of ArcticAβ mice to a seizure inducing drug is an intriguing outcome of this project, suggesting alterations in synaptic function or in intrinsic properties of neurons in mutant mice. Overexcitability could potentially be related to the pathological changes in inhibitory system, as a result of APP overexpression that could affect the development of circuitry or the production of toxic Aβ species at the ages when mice received KA treatment. The exact mechanisms and prevention of such excitability requires further investigation.

As discussed before, ArcticAβ mice that survive KA injection might represent a group of animals with delayed AD pathology development and/or coincident "mild" KA lesion phenotype due to variations in injection technique. Being a remarkable outcome, it is also a pitfall of this experiment, as it makes it difficult to conclude the effects of seizures on AD pathology. A potential solution for this problem, from AD model perspective, would be extending the experiment to later timepoints, as looking beyond 6 months post KA, could reveal if the survivors represent the group with delayed AD pathology. On the other hand, using transgenic AD mouse models that develop AD pathology faster could speed up the obtainment of the results. In combination with this, extending diazepam treatment to the first weeks post KA injection could also potentially help prevent the mortality. Also, alternatively to KA model, more controlled approach towards seizure induction, such that involves for example optogenetics could be used (Krook-Magnuson et al., 2015). Abberant neuronal activity was shown in other FAD mouse models (Palop et al., 2007, Westmark et al., 2008). In the next chapter, we evaluated spontaneous seizure activity in ArcticAβ mice aged up to 15 months. Prospectively, correlating spontaneous seizures with the degree of AD pathology in the same animals could give us an idea if endogenous seizures could aggravate AD pathology.

CHAPTER II: ADULT NEUROGENESIS IN ARCTICAβ MICE

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Part of a submitted publication

Author's contribution

All the experimental procedures were carried out by MZ under the supervision by JMF. MZ wrote the manuscript. Authors have no conflict of interest to declare.

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Abstract

Our previous work showed that ArcticAβ mice, which overexpress human APP with double Swedish and Arctic mutations, are hypersensitive to intra-hippocampal injection of kainic acid (KA), used to model temporal lobe epilepsy. Here, we tested the hypothesis that altered adult neurogenesis in these transgenic mice contributes to their hyperexcitability in response to convulsants, and thereby seizure propensity in familial AD-mutant mice. Using pulse labeling with BrdU, we observed that ArcticAβ mice have increased precursor cell proliferation, but reduced cell survival in the subgranular zone of the hippocampal dentate gyrus, having no net effect on the generation of new neurons. As evidenced by retrovirally-mediated eGFP expression, we observed that adult born neurons in ArcticAβ mice have decreased number of dendritic spines and reduced dendritic branching during their maturation. Therefore, disturbances in dendritic maturation might affect the integration of newborn neurons in hippocampal networks, implying that the net inhibitory effect of adult neurogenesis would be weakened. This alteration could possibly contribute, along with other factors, to the higher sensitivity of ArcticAβ mice to seizure-inducing drugs.

Introduction

ArcticAβ mice (Knobloch et al., 2007) are highly sensitive to Kainic acid induced seizures (Chapter 1). Unilateral KA injection into the dorsal hippocampus caused severe seizures and increased mortality in this mouse model in the first weeks after KA treatment. Sensitivity to seizure inducing drugs is not uncommon among FAD mouse models and there is an ongoing search for commonalities in those models that can trigger such reactivity.

Recent studies showed that adult neurogenesis is involved in seizure susceptibility in multiple epileptic mouse models. Suppression of neurogenesis can increase the acute effect of KA in mice (Iyengar et al., 2015), however, the opposite effect was observed in pilo-carpine epileptic model (Cho et al., 2015). Adult neurogenesis was previously shown to be affected in a number of AD mouse models (Sun et al., 2009, Biscaro et al., 2012, Wang et al., 2014) and human patients (Jin et al., 2004a). Adult-born neurons were previously shown to have a net inhibitory effect on the DG-CA3 network (Acsady et al., 1998). Also, it was shown that suppression of adult neurogenesis can increase the acute effects of KA in mice (Iyengar et al., 2015).

Taking these evidences together, we hypothesized that changes in adult neurogenesis in the ArcticAβ mice could underlie their increased sensitivity to KA. The series of experiments in this project will show whether extensive hAPP overexpression and an increase in toxic intracellular Aβ in ArcticAβ mice affect their adult neurogenesis. This study can contribute to our understanding of the underlying mechanisms of KA induced seizure susceptibility in this mouse model.

To get basic insights into changes in neurogenesis in ArcticAβ mice we used 5-bromo-2'-deoxyuridine (BrdU) treatment to look at the proliferation and survival rates and fate commitment of newborn cells in the hippocampal subgranular zone. Further, we used intrahippocampally injected retrovirus that triggers GFP expression under CAG promoter to label newborn cells to analyze the dendritic complexity and morphology and density of spines of those cells that were committed to become neurons.

Materials and Methods

Animals

All experiments performed for this study were carried out in accordance with Swiss law on animal experimentation and approved by the Cantonal Veterinary Office of Zurich. Experiments were performed with ArcticAβ transgenic mice (Knobloch et al., 2007) and age-matched wild-type littermates. All animals were housed in groups of 2-5 in individually ventilated cages at standard conditions (20-24 °C; minimum 40% relative humidity) under a 12-hour light/dark cycle, with access to food and water ad libitum.

Bromodeoxyuridine (BrdU) treatment

To avoid estrous cycle related effects on adult neurogenesis (Pawluski et al., 2009, Marques et al., 2016), three weeks prior BrdU treatment adult female mice were housed together at highest density permitted (5 per cage) and bedding containing male mouse urine was introduced to the cages to induce Lee-Boot and Whitten cycle-synchronizing effects respectively (Caligioni, 2009, Byers et al., 2012). At the age of two months on two consecutive days mice received two intraperitoneal injections of 90 mg/kg BrdU (Sigma-Aldrich, #B5002, dissolved in 0.9% NaCl). The tissue was collected at 1 dpi to assess cell proliferation and at 28 dpi to evaluate cell survival and cell fate.

Intrahippocampal virus injection

Adult male mice (2-3 months old) were anesthetized by inhalation of 2.5-3% isoflurane (Baxter) in oxygen. Retrovirus encoding enhanced green-fluorescent protein (eGFP; total injection volume per side - 1 µl) was bilaterally injected (Nanoject II, Drummond Scientific) into the hilus of the dentate gyrus (stereotactic coordinates: antero-posterior: -2.0 mm, lateral: +/- 1.5 mm, dorso-ventral: - 2.3 mm relative to Bregma). During surgery and recovery mice were kept on a warm pad. After the surgery mice were given an intraperitoneal injection of analgesic buprenorphine (Temgesic, Essex Chemicals).

Tissue preparation for immunohistochemistry

Brain tissue was collected at 21 and 42 days post injection (dpi). Tissue was collected and prepared following the protocol of Notter et al. (2014). In brief, mice were anesthetized (Nembutal; 50 mg/kg; i.p.) and perfused intracardially through the left ventricle with 20-25 mL of ice-cold, oxygenated aCSF [containing (mM) NaCl 125, KCl 2.5, CaCl2, 3.7, MgCl2 2,

NaHCO3 26, NaH2PO4 1.25, glucose 25], pH 7.4, at a flow rate of 10–15 mL/min. Brain was extracted and a block containing hippocampus was immediately immersion-fixed for 3 hours in ice-cold fixative [4% paraformaldehyde dissolved in 0.15M sodium phosphate buffer, pH 7.4). Tissue was then rinsed with PBS (pH = 7.4) and cryoprotected overnight in 30% sucrose in PBS at 4°C.

Fixed and cryoprotected tissue was sectioned into 40µm ($BrdU^+$ experiment) or 70µm (eGFP experiment) thick serial coronal sections using tissue mounting fluid (M-1 Embedding matrix, Shandon, Thermo Scientific, USA) and freezing sliding microtome (MICROM HM 400, MICROM International GmbH, Walldorf, Germany) at -40°C and collected into ice-cold PBS. For long-term storage, sections were transferred into cryoprotectant solution (50mM sodium phosphate buffer, pH 7.4, containing 15% glucose and 30% ethylene glycol; Sigma-Aldrich) and kept at -20°C.

Immunohistochemistry

For immunofluorescence, sections were washed three times in PBS (pH 7.4, 10 min each) and incubated overnight (BrdU experiment) or for 72 hours (eGFP experiment at 4°C under continuous agitation with primary antibodies diluted in Tris buffer (50mM Tris, 150mM NaCl, 0.05% Triton X-100, pH 7.4) containing 2% normal goat serum (NGS) or normal donkey serum (NDS) and 0.2% Triton X-100. Afterwards sections were rinsed three times in PBS and incubated in secondary antibody solution (2% NGS, Tris buffer) for 30 min (BrdU experiment) or 6 hours (eGFP experiment) in darkness, at room temperature, under continuous agitation, with secondary antibodies raised in goat. Secondary antibodies conjugated to AlexaFluor-488 (Invitrogen) were diluted to 1:1000 and those conjugated to Cy3 or Cy5 (Jackson ImmunoResearch Laboratories) to 1:500. After washing, three times with PBS, sections were mounted on gelatin-coated glass slides and cover-slipped using Dako Fluorescence Mounting Medium (Agilent Technologies, Santa Clara, CA, USA). Tissue was kept in the dark at 4°C prior imaging.

Target	Description	Dilution	Distributor	Procedure
Primary				
BrdU	rat	1:5000	Oxford Biotech	IF, IP
NeuN	mouse	1:1000	Chemicon	IF
GFAP	rabbit	1:20000	Dako	IF
GFP	chicken	1:5000	Aves Labs Inc.	IF
Secondary				
rat	donkey, Alexa 488-conjugated	1:1000	Jackson Immuno Research Inc.	IF
mouse	donkey, Cy3-conjugated	1:500	Jackson Immuno Research Inc.	IF
rabbit	donkey, Cy5-conjugated	1:500	Jackson Immuno Research Inc.	IF
chicken	goat, Alexa 488- conjugated	1:1000	Jackson Immuno Research Inc.	IF

Table 1. List of primary and secondary antibodies used for immunohistochemistry.

Making BrdU available to primary antibodies required pre-treatment prior immunoperoxidase or immunofluorescence staining. Free-floating sections were incubated in 0.2N HCl for 5 minutes at room temperature, before being transferred into 4N HCl for 30 minutes at 37°C. Right afterwards and before immunostaining, sections were rinsed twice in PBS and twice in Tris-Triton. For immunoperoxidase staining, biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories) were diluted 1:300 and after washing three times in Tris buffer, incubated with avidin-peroxidase-complex solution (Vectastain Elite kit, Vector Labs) at room temperature. After washing three times in Tris buffer, sections were stained by combining 3,3-diaminobenzidine (DAB; Sigma–Aldrich Inc.) in Tris buffer (pH 7.7) with hydrogen peroxide for 5-15 min. Sections were immediately transferred to ice-cold PBS and washed three times. Finally, sections were mounted onto gelatinized glass slides, air-dried overnight, dehydrated with ethanol, cleared in xylene and cover-slipped with resinous (Eukitt™; Sigma-Aldrich) mounting medium.

Image acquisition and analysis

Stereological analysis

$BrdU^+$ cells in the subgranular zone (SGZ) and granule cell layer (GCL) of the dentate gyrus were counted in 4-5 sections per mouse in dorsal hippocampus using Axioscop, (Zeiss, Jena, Germany) 40x oil-immersion lens, NA 1.3. Dorsal hippocampal volume was estimated using Mercator software (Mercator Pro rev. 7.8.2, Explora Nova, La Rochelle, France). $BdrU^+$ cells in the DG SGZ were quantified manually.

Sholl analysis

Fluorescent Z-stacks (spaced by 0.7 μm) throughout the entire thickness of the section were acquired with were acquired with confocal microscope LSM710 using 40x (NA, 1.4) oil immersion objective and ZEN 2012 black edition (Carl Zeis MicroImaging GmbH, Goettingen, Germany) software. Images were analysed with ImageJ (version 1.49o; Java 1.6.0_12 (Wayne Rusband, National Institutes of Health, USA). Sholl analysis (Sholl, 1953) was used to analyze the complexity of the dendritic trees. Complexity if dendritic tree was equivalent to the number of intersection of dendrites with concentric circles spaced by 10μm intervals, centered on the cell body. First, neurons were traced using NeuronJ plugin (NIH ImageJ; Meijering (Meijering et al., 2004)). Further Sholl analysis macro (Anirvan Ghosh Laboratory, University of California, San Diego, La Jolla, CA, USA) was used to calculate the numbers of intersections between dendrites and concentric circles. For the number of intersections as a function of distance from the soma, area-under the curve (AUC) was calculated and used for further statistical analysis. Dendritic morphometry (primary dendrite length, total dendrite length) was assessed with the NeuronJ plugin. In total 14-31 cells from the groups of 5 to 8 mice per time point were analyzed.

Spine density analysis

Z-stacks for spine density and morphology analysis of randomly selected eGFP-positive dendritic segments were acquired with 40x oil immersion objective using 2.7 digital zoom factor. Spines density quantification and manual spine type classification were performed using ImageJ plugin Cell Counter. No distinction was made between spine and filopodia. The length of each segment was measured and the number of spines/μm was quantified with Excel.

Statistical analysis

Data is shown as mean ± standard error of the mean (s.e.m.). An unpaired, two-tailed, Student's t-test was used to compare two groups. Statistical analysis and graphs were plotted with Prism

software (GraphPad Software Inc., La Jolla CA, USA, version 6). Figures were designed with Abobe Illustrator CSS 15.1.0.

Results

Proliferation and survival of adult newborn cells are affected in ArcticAβ mice

BrdU labelling experiments were performed in adult ArcticAβ mice and wild-type controls. BrdU ip injections were performed on two consecutive days and tissue was collected at 1 and 28 dpi (days post injection) (Figure 1.a). Collected brain tissue was further immunostained for BrdU and $BrdU^+$ cells were stereologically quantified. At 1 dpi there was a significant increase of $BrdU^+$ cell density in the subgranular zone of the hippocampal dentate gyrus of ArcticAβ compaired to wild-type mice (14039 ± 1243 cells/mm^3, n=8 10415 ± 728 cells/mm^3, n=8; data shown as mean ± s.e.m.; P=0.821, paired, two-tailed Student's t-test). This indicates higher NPC proliferation rate in ArcticAβ mice (Figure 1.b,c). At 28 dpi there was no difference observed between the $BrdU^+$ cell density in the SGZ between the genotypes (ArcticAβ: 2663 ± 261.8 cells/mm^3, n=9; wild-type: 3384 ± 491 cells/mm^3, n=9; data shown as mean ± s.e.m.; P=0.214, paired, two-tailed Student's t-test), suggesting a decreased survival rate of NPC in ArcticAβ mice (Figure 1.b,c). Increased NPC proliferation is consistent with some of the earlier reports from FAD mouse models, specifically those that also overexpress APP (Jin et al., 2004a, Gan et al., 2008) and with data from AD patients (Jin et al., 2004b). Long-term survival of NPCs was reported decreased in several hAPP transgenic lines and in AD brains (Haughey et al., 2002, Boekhoorn et al., 2006, Donovan et al., 2006, Verret et al., 2007, Li et al., 2008). Increased proliferation could be ascribed to the increased levels of soluble APP (sAPP) that was shown to regulate NPC proliferation (Demars et al., 2011, Lazarov and Demars, 2012, Demars et al., 2013) and/or to the compensatory mechanisms due to the elevated toxic Aβ species in neurons at this age of animals.

Neuronal vs astrocytic fate commitment was not affected in ArcticAβ mice with 89-91% being positive for neuronal marker NeuN, 6-7% being positive for astrocytic marker GFAP and about 3% not labelled for either NeuN or GFAP (commited to become microglia) (Figure 1.d). Similar ratios were reported in previous studies from our lab for WT (Duveau et al., 2011a).

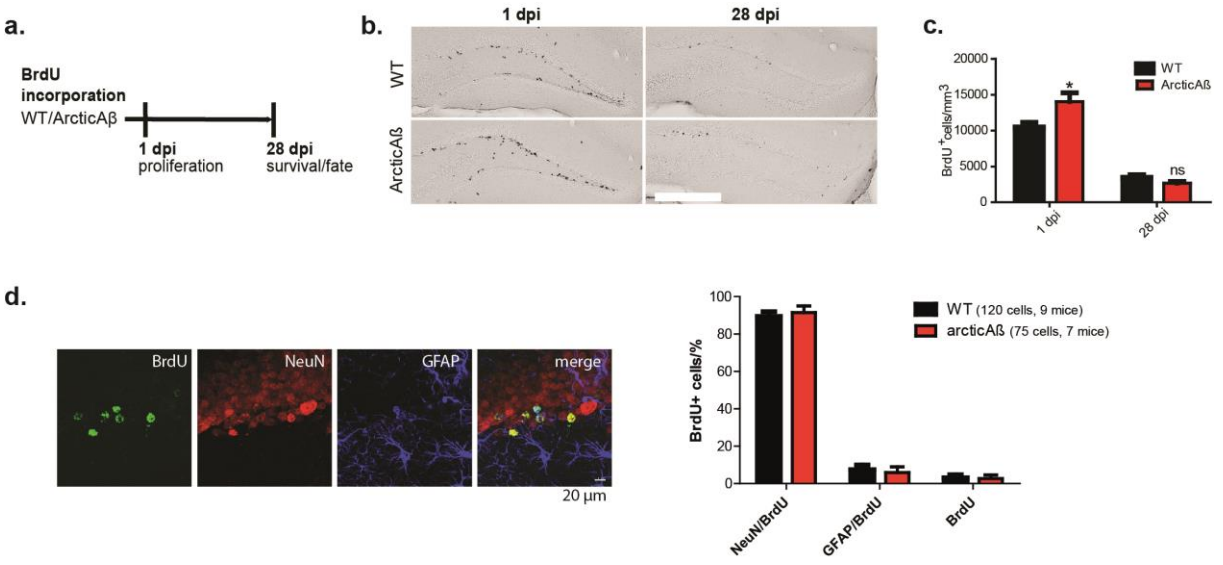

Figure 1. Differences in newborn cell proliferation and survival between wild-type and ArcticAβ mice.

(a) Schematic representation of the experimental design. Mice received BrdU injections on two consecutive days and brain tissue was collected at day 1 and 28 post BrdU injection to evaluate cell proliferation and survival rate in the DG respectively. **(b)** Representative images of BrdU immunostaining 1 and 28 days after the second BrdU treatment. $BrdU^+$ cells were located in the inner part of the GCL. Higher density of $BrdU^+$ cells was common in ArcticAβ mice at 1 dpi, but there were no differences at 28 dpi between two genotypes (scale bar - 160µm). **(c)** Unbiased quantification of $BrdU^+$ cells in both genotypes. ArcticAβ mice have a significantly increased proliferating cell density at 1 dpi. Density of $BrdU^+$ cells was not different between two genotypes at 28 dpi. (**P*>0.05, unpaired t-test). **(d)** Triple immunofluorescent staining for BrdU, NeuN and GFAP to differentiate $BrdU^+$ neurons and astrocytes showed no difference in neurogenesis vs gliogenesis in ArcticAβ mice.

Dendriric arborization is reduced in ArcticAβ mice

Next, we investigated dendritic arborization of newborn granule cells using retroviral labeling technique. eGFP- encoding retrovirus was stereotactically injected in the dorsal hippocanmpus of wild-type and ArcticAβ adult mice. The virus incorporates into the dividing cells and thus the day of the injection marked the birthday of the neuron. Brain tissue was collected for further analysis at day 21 and 42 post-injection and immunostained for signal enhancement for eGFP for further confocal imaging (Figure 2.b.). Neurons were readily discriminated from dividing astrocytes and microglia by their distinct morphology and location. 21 and 42 dpi cover immature and mature stages of neuronal development (Figure 2a.). Complexity of dendritic arborization was estimated using Sholl analysis (number of intersections with concentric circles centered on the soma). There was overall tendency towards reduced dendritic arborization at both 21 and 42 dpi in ArcticAβ mice, however further away from soma, starting from 150µm this difference was statistically significant (at 21 dpi wild-type: 197.4 ± 31.29, N=27; ArcticAβ:

69.64 ± 23.31 N=14; P=0.0094, at 42 dpi wild-type: 359.0 ± 24.77 N=31, ArcticAβ: 257.7 ± 33.91 N=15, P= 0.0221; data displayed as area under the curve (AUC) mean ± s.e.m., paired, two-tailed Student's *t*-test) (Figure 2.c,d).

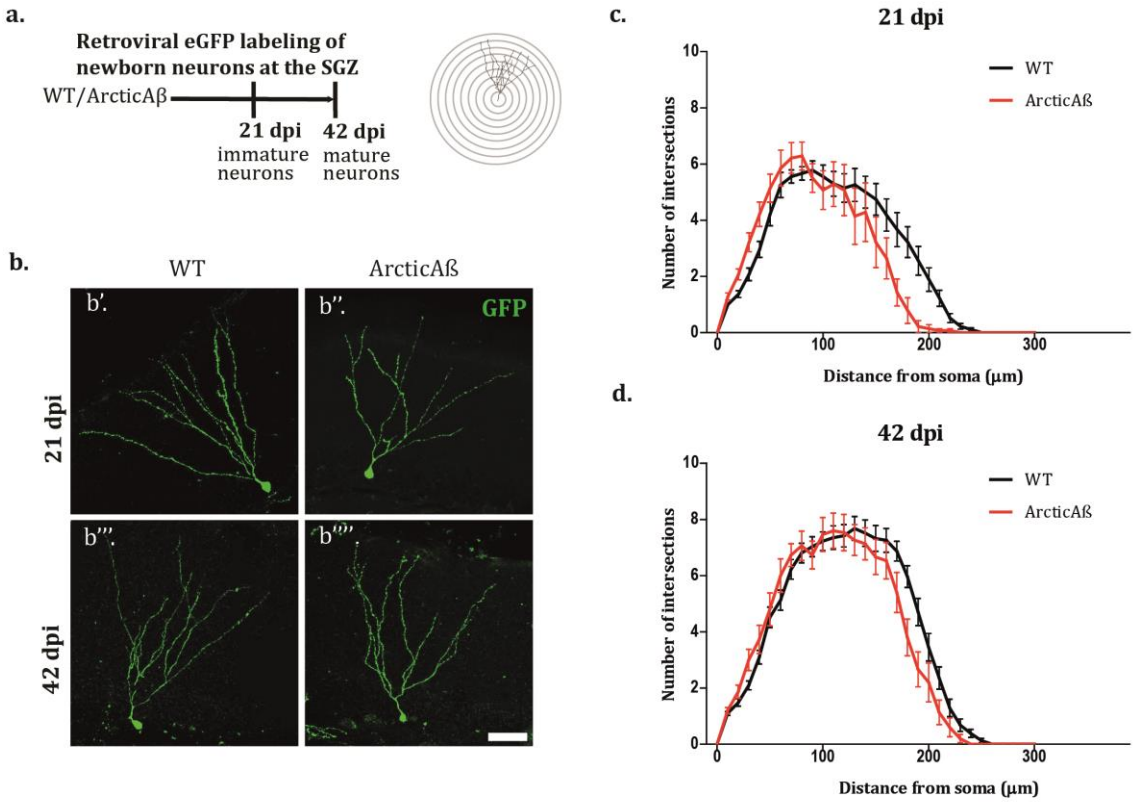

Figure 2. Reduced dendritic arborization in ArcticAβ newborn neurons.
(a) Schematic representation of the experimntal design. Brain tissue of both genotypes was collected 21 and 42 days after the intrahippocampal injection of eGFP retrovirus (left). Quantification of dendrtitic arborization of eGFP+ adult-born neurons was done by Sholl analysis that measures the number of intersections between eGFP+ dendrites and virtual concentric circles centered on the cell body and spaced by 10µm (right). **(b) (b'-b'''')** Fluorescent images of representative examples of eGFP+ cells in each genotype at 21 **(b',b'')** and 42 **(b''',b'''')** dpi (Scale bar – 50 µm). **(c,d)** Quantitative result of Sholl analysis showed no significant difference, but a trend towards reduced dendritic arborisation in ArcticAβ at 21 dpi in comparison to wild-type. There were no differences between genotypes at 42 dpi.

Spine density is reduced in ArcticAβ mice

Further, we analyzed overall spine density and spine morphology between wild-type and ArcticAβ mice at 21 and 42 dpi. Mushroom, stubby and thin spines correspond to immature and mature types respectively. Overall and mature type (thin) spine density were reduced in ArcticAβ mice at 21 dpi, indicating abnormal neuronal maturation. This difference was not present at 42 dpi, where only the density of stubby spines was reduced in mutant mice,

suggesting possible delayed development of newborn neurons in ArcticAβ mice (Figure 3.a,b; Table 1). There was no significant difference in proportions of spine types at 21 (wild-type – thin 76%, stubby 15%, mushroom 9%; ArcticAβ – thin 74%, stubby 17%, mushroom 9%) and 42 dpi (wild-type – thin 65%, stubby 26%, mushroom 9%; ArcticAβ – thin 71%, stubby 21%, mushroom 8%) (Figure 3.c).

Our results go in line with previous research on a similar FAD mouse model that exhibited abnormal morphological development at later neuronal developmental stages (Sun et al., 2009). Our experiments were performed in 2-3 months old mice. At this age, there are still no amyloid plaques accumulated, but Aβ is shown to already increase intracellularly (Knobloch et al., 2007). hAPP-I5 mice that have high hAPP levels, but relatively low Aβ levels exhibit normal adult-born GC development (Mucke et al., 2000). Taking this into account, it's likely that in ArcticAβ mice elevated Aβ levels, but not hAPP overexpression could be held responsible for the abnormalities in dendritic arborization, Sun et al. showed that dendritic spine density was increased at early developmental stages, but went down in hAPP-J20 mice after 28 dpi. hAPP-J20 mice overexpress hAPP with Indiana and Swedish mutations (Mucke et al., 2000). The latter they share with ArcticAβ mice, that additionally have hAPP Arctic mutation. The role of hAPP mutations could also not be completely excluded and could explain the differences in adult

21 dpi	**WT**	**ArcticAβ**	**significance, *p* value**
Mushroom	0.059 ± 0.006, n=27	0.044 ± 0.008, n=14	not significant, 0.164
Stubby	0.102 ± 0.016, n=27	0.076 ± 0.016, n=14	not significant, 0.298
Thin	0.508 ± 0.037, n=27	0.316 ± 0.032, n=14	significant, 0.002
All	0.669 ± 0.511, n=27	0.435 ± 0.044, n=14	significant, 0.005
42 dpi	**WT**	**ArcticAβ**	**significance, *p* value**
Mushroom	0.096 ± 0.010, n=18	0.084 ± 0.009, n=18	not significant, 0.415
Stubby	0.301 ± 0.037, n=18	0.220 ± 0.015, n=18	significant, 0.052
Thin	0.728 ± 0.057, n=18	0.732 ± 0.062, n=18	not significant, 0.965
All	1.126 ± 0.085, n=18	1.037 ± 0.069, n=18	not significant, 0.419

Table 1. Spine density and statistical analysis.
Numerical data of mushroom, stubby, thin and overall spine density (spines per µm) in wild-type and ArcticAβ mice at 21 and 42 dpi. Data displayed as mean ± s.e.m. There were significantly more thin and generally all spines in wild-type vs ArcticAβ mice at 21 dpi. At 42 dpi there was significant difference in stubby spines in wild-type vs ArcticAβ. Statistical test - paired, two-tailed Student's *t*-test.

neurogenesis in those models. Arctic mutation promotes higher rate of $A\beta_{40}$ protofibril formation (Nilsberth et al., 2001). Indiana mutation leads to an increase of $A\beta_{40}/A\beta_{42}$ ratio (Tamaoka et al., 1994). Differences in the amount of more toxic Aβ species and/or increased Aβ40 toxicity, could account for those differences in spine development.

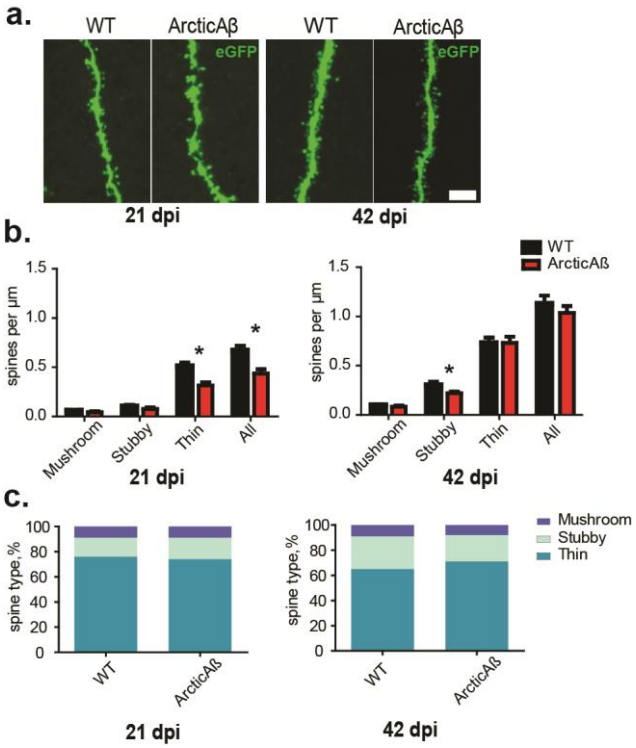

Figure 3. Reduced spine density in newborn neurons in ArcticAβ mice.
Dendritic spine classification analysis. **(a)** Representative images of dendritic segments from eGFP expressing GCs of wild-type and ArcticAβ mice at 21 and 42 dpi (Scale bar – 5 µm). **(b)** Quantification of mushroom, stubby, thin and overall spine density. ArcticAβ mice had significantly reduced overall spine density and specifically thin spine density at 21dpi and reduced stubby spine density at 42 dpi with no difference in overall spine density. **(c)** There were no significant differences between the proportions of all three types of spines between the two genotypes.

Conclusion

Using BrdU labelling we found that ArcticAβ mice have increased proliferation rate, but decreased survival of NPCs. Neurogenesis vs gliogenesis cell fate was not affected in the transgenic animals. This result is consistent with many reports from other FAD mouse models and also from humans (Jin et al., 2004a, Jin et al., 2004b, Gan et al., 2008). FAD mouse models that overexpress human APP, ArcticAβ mice included, tend to have have increased NPC proliferation, which is probably related to high amounts of soluble APPα (sAPPα), a proteolyte of APP processing. sAPPα was reported to increase adult projenitor cells proliferation (Demars et al., 2011, Demars et al., 2013).

Next, with viral eGFP labeling of newborn cells we showed that ArcticAβ mice have decreased dendritic tree complexity and have decreased spine density during maturation in comparison to wild-type. This morphological result is for the most part consistent with what has been shown for hAPP-J20 FAD mice (Sun et al., 2009). In this study, the authors used electrophysiological techniques to show that disbalance of GABAergic and glutamatergic inputs in the mouse model is the reason for the alterations in the development of the newborn neurons. hAPP-J20 and ArcticAβ mice have double Swedish mutation in common, which is immediately adjacent to the β-secretase site in APP sequence and results in increase of total Aβ levels. It would be interesting to check if impairments in neurogenesis could be rescued with antiAβ antibodies treatment. Adult-born neurons were suggested to have a net inhibitory effect on the DG-CA3 network, by extensive innervation of interneurons (Heinemann et al., 1992, Lothman et al., 1992, Acsady et al., 1998, Hsu, 2007, Ikrar et al., 2013, Iyengar et al., 2015). Alterations that occur during maturation in adult born neurons in ArcticAβ mice can lead to inappropriate circuit integration and lead to weakening of the overall inhibition in DG-CA3 network, which could decrease seizure threshold in ArcticAβ mice.

CHAPTER III: MECHANISMS OF INHIBITORY SYNAPTIC STRENGTHENING AT CEREBELLAR STELLATE INTERNEURONS

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In preparation for submission

Author's contribution

All the experimental procedures were carried out by MZ under the supervision of DB. EL is continuing the project. MZ and JMF wrote the manuscript. Authors have no conflict of interest to declare.

Abstract

Dysfunction of interneurons underlies a variety of CNS disorders, including Alzheimer's disease and epilepsy. GABAergic interneurons control the firing and synchronization of principal neurons, thereby driving network oscillatory activity. This study expands our understanding of a unique inhibitory synapse strengthening mechanism, first observed in cerebellar stellate interneurons and triggered by mitochondrial reactive oxygen species (mROS). mROS is traditionally associated with oxidative stress and its harmful effects. In addition, however, mROS plays an important physiological role in signaling. The production rate of mROS reflects dynamic metabolic demand of a neuron, and, therefore its level of activity. mROS triggers a cascade of signaling events that lead to strengthening of inhibitory transmission by recruiting more $GABA_A$ receptors ($GABA_AR$) at postsynaptic sites, providing a protective feedback mechanism in case of overactivation. Interestingly, the recruited receptors have distinct biophysical properties compared to the predominant resident receptor type, due to a different subunit composition. In this study, we further explored this form of plasticity and demonstrate that repeated high frequency stimulation (HFS) of input fibers, used to strongly activate stellate cells in brain slices, leads to un-silencing of a distinct pool of $GABA_ARs$ in stellate cells. Such inhibitory postsynaptic currents IPSCs with distinct kinetic properties could be detected by comparing evoked responses to a minimal stimulation before and after HFS, suggesting recruitment of additional $GABA_ARs$ to postsynaptic sites. We show that the mechanism of this recruitment is likely postsynaptic and involves mROS, since the effect of HFS could be strongly attenuated by an antioxidant present in the recording pipette. Interestingly, the effect of HFS was variable among the recorded stellate cells, suggesting that different types of responses might correspond to different origin of synaptic inputs onto stellate cells. The next steps will be to identify the exact signaling pathways that underlie this synaptic strengthening, in order to mimic their action pharmacologically, and to identify endogenous stimuli or molecules inducing this form of plasticity.

Introduction

Neuronal synapses play a central role in signal transmission and transduction in the CNS. Their variety and the wide range of mechanisms by which they can be modified form the basis of neuronal plasticity. There are synapses that under basal conditions are not taking part in neurotransmission and form a "reserve pool" that can be activated under specific circumstances. Almost two decades ago, so called silent synapses were first described in the CA1 area of the hippocampus. (Isaac et al., 1995, Liao et al., 1995). These synapses are not eliciting excitatory postsynaptic currents (EPSCs) at the resting membrane potential, but do so upon depolarization. AMPARs are conducting currents at the resting membrane potential. Activation of NMDARs requires depolarization to remove magnesium block. Glutamatergic synapses are „silent" if they contain only NMDARs and no AMPARs; under physiological conditions are not mediating synaptic transmission. Synapses become un-silenced upon application of high frequency long-term potentiation-inducing stimulation in combination with depolarization; AMPA receptors are possibly trafficked to those synapses under these conditions. Since the discovery of silent synapses, their role in synaptic plasticity and strengthening, as well the physiological mechanisms of un-silencing were extensively studied. Silent synapses were subsequently described in many other brain regions (Kerchner and Nicoll, 2008).

Silent synapses are not necessarily excitatory. Paired recordings of goldfish brainstem Mauthner cells, and their presynaptic partners, local glycinergic interneurons, revealed that transmission failed to occur in many cases in response to external stimulation. Series of titanic stimulation un-masked these synapses, providing evidence for one type of long-term potentiation at inhibitory synapses (Korn et al., 1992).

Rebound potentiation at the cerebellar Purkinje cells (PCs) is another classical example of inhibitory synaptic plasticity in the CNS (Kano et al., 1992). It is characterized by an increase in inhibitory postsynaptic currents (IPSCs) amplitude following PC depolarization. This type of synaptic plasticity relies specifically on β2-$GABA_A$ receptors that are predominant at somatic PC synapses originating from basket cells (BCs) and on the activity of CAMKII. Rebound potentiation is an important regulator of PC spontaneous firing and is crucial for temporal coding of PC output and the level of inhibition of deep cerebellar nuclei (Hirano et al., 2016).

Recent research has shown that elevation of mitochondrial reactive oxygen species (mROS) can also lead to strengthening of inhibitory transmission at GABAergic stellate interneurons in the cerebellar molecular layer, apparently by increasing the number of functional GABAergic synapses by a postsynaptic mechanism. The rate of mROS production is reflecting the metabolic state of the cell. Increased neuronal firing elevates mROS and can lead to the recruitment of new receptors to the synapse. α1 subunit-containing $GABA_A$Rs were long thought to be the predominant subtype of $GABA_A$Rs expressed in stellate cells; however, there is strong evidence, that mROS mediates synaptic strengthening by indirectly recruiting α3 subunit-containing $GABA_A$Rs to the postsynaptic membrane (Accardi et al., 2014). Underlying mechanisms of this process are still being investigated.

The aim of this study is to investigate whether similarly to silent synapses, a synaptic reserve pool containing α3-$GABA_A$Rs can also be un-silenced under specific conditions. We stimulated input fibers in the vicinity of recorded stellate cell with high frequency stimulus (HFS). Before and after a series of HFS we recorded evoked IPSCs in a stellate cell using minimal stimulation approach (Isaac et al., 1995) to demonstrate the un-silencing of the synapses. The intensity of minimal stimulation for each recording is established before the series of HFS and is supposed to evoke 20-50% of IPSCs. After the un-silencing stimulus (HFS in this case) we looked at the changes in the number of evoked IPSCs and their kinetics. HFS puts a cell into a high energy demanding mode, which can trigger mROS production. We expect to see the rise in evoked IPSCs after a series of HFS.

Insulin is known to increase mROS in neurons, by which mechanism it can induce synaptic strengthening at cerebellar granule cells through activating a cascade of events that recruits α6-$GABA_A$Rs to postsynaptic sites, where α1-$GABA_A$Rs are predominant (Accardi et al., 2015). We were curious to test whether this mechanism can be generalized to other cell types and might lead to strengthening inhibitory transmission in stellate interneurons as well. Thus, we tested the effect of insulin by whole-cell patch clamp recording of miniature IPSCs in stellate cells.

Materials and Methods

Animals

Wild-type mice with C57BL/6J genetic background (Charles River laboratories) were maintained as a breeding colony at McGill University (Montreal, Quebec, Canada). Both males and females were used for experiments. α3-KO mice were maintained at C57BL/6J background (B6.129 1-Gabra3tm2Uru/Uru) and bred at McGill University. Only α3-KO male mice were genotyped by PCR analysis of tail biopsies and further used in the experiments. The age of animals used in those experiments varied between 15 and 28 days. All experiments have been approved by the local authorities and were performed in accordance with the guidelines of the Canadian Council on Animal and were approved by the Animal Care Committee of McGill University (Protocol Number: 4564).

Cerebellar slice preparation

Mice were anesthetized with Isoflurane and immediately decapitated. Cerebellum was rapidly detached from the brain and extracted into an ice-cold oxygenated (95% O2,5% CO2) cutting solution, containing (in mM): 235 sucrose, 2.5 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 7 MgSO4 and 28 D-glucose (pH of 7.4; osmolarity of 305– 315 mOsmol/L). Cerebellar tissue was fixed on the stage of the vibrating tissue slicer (Leica VT1200) cutting chamber with medical glue. The cutting chamber was filled with the ice-cold cutting solution, which was oxygenated (95% O_2, 5% CO_2) during the entire slice preparation procedure. Each 300 μm thick cerebellar vermis slice was immediately transfered into oxygenated, room temperature (20–23 °C) artificial cerebrospinal fluid (aCSF) that contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 1 MgSO4 and 25 D-glucose (pH of 7.4; 305–315 mOsmol/L). Recordings started not earlier than 1 hour after the slice preparation.

Electrophysiology

Whole-cell patch clamp recording were performed on the stellate interneurons, visually identified according to their cell body size (8-9 μm) and location in the outer two-thirds of the molecular layer. Recordings were obtained on two electrophysiological setups, equipped with an Olympus BX51 upright microscope (Olympus) both utilizing differential interference contrast/infrared optics. Patch pipettes were prepared from thick-walled borosilicate glass (GC150F-10, OD 1.5 mm, ID 0.86 mm; Harvard Apparatus) with PC-10 pipette puller (Narishige). Patch pipettes has open tips resistances of 5-10 MΩ when filled with intracellular

solution that contained (in mM): 140 CsCl, 4 NaCl, 0.5 CaCl2, 10 HEPES, 5 EGTA, 2Mg-ATP, 2 QX314 to block voltage-activated Na+ channels (pH of 7.4 adjusted with CsOH, osmolarity of 300–310 mOsmol/L).

Recordings were obtained with Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA) at a holding potential of -60mV. Series resistance and whole-cell capacitance were estimated by cancelling the fast current transients evoked at the onset and offset of brief (10–20 ms) 5mV voltage-command steps. Series resistance (10–35 MΩ) was compensated at 40% and monitored for stability throughout the experiments. Currents were filtered at 5 kHz with an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA, USA) and digitized at 25 kHz with a Digidata 1322A data acquisition board and Clampex9 (Molecular Devices). The capacitance of the stellate cells was in the range of 6–11 pF. The bath was continuously perfused with aCSF at room temperature (22–23°C) at 1–2 ml/min flow rate. Minimal stimulation (MS) experiments were performed in the presence of GYKI to block AMPA receptor currents. mIPSCs were recorded in the presence of TTX (1 mM) in the aCSF.

For extracellular stimulation aCSF filled thin-walled borosilicate glass electrodes (OD 1.65 mm, ID 1.15 mm; King Precision Glass Inc., Claremont, CA, USA) with a tip current of < 3 MΩ were used. A platinum wire wrapped around the stimulation electrode served as the ground electrode for the stimulation circuit. After a stable whole-cell patch was obtained, the stimulating electrode was positioned in the outer two-thirds of the molecular layer beneath the slice surface, 50 to 100 μm away from the cell body. Voltage pulses (15-30 V in amplitude, 200–400 ms in duration) were applied at low frequency (0.1 Hz) through the stimulating electrode to identify if the stimulation is evoking IPSCs.

The voltage intensity used, was the lowest necessary to obtain 100% success (I_{100}) in evoking IPCSs. Stimulation voltage intensity was lowered until the success rate was in the range of 25-75% and this value would be noted (I_{50}) as the intensity for minimal stimulation (MS) protocol. GYKI was washed in with the perfusing aCSF, which was followed by two control 5 minute long recordings at 0.1Hz at I_{100}. If the evoked events visibly decreased (about 50%) due to the action of GYKI, it would confirm that the interneuron has preserved glutamatergic inputs and this cell would be further used in the experiment. Minimal stimulation lasted five minutes at 0.5 Hz frequency with I_{50}, at V_m = -60 mV. First control MS recording was followed by high frequency stimulation (HFS) (6 pulses at 100 Hz repeated 3 times in 1 minute) at I_{100} at V_m = +40 mV. Series of alternating HFS and MS were repeated five times. Recording time for each experiment lasted 45 minutes.

Analysis

Curve fitting and figure preparation of all electrophysiology data were performed with Origin 7.0 (OriginLab), Microsoft Excel, Clampfit 10 (Molecular Devices). The decay phase of evoked IPSCs were fit with a single exponential curve, mIPSCs were analyzed with Strathclyde Electrophysiology WinWCP and WinEDR (John Dempster) software. mIPSCs detection threshold was of four times the mean root square noise level. Detection of mIPSCs was visually confirmed for quality control. All data are expressed as mean ± s.e.m.; P-values represent the results of paired, two-tailed Student's t-tests. A P-value of less than 0.05 was considered statistically significant.

Pharmacological compounds

GYKI 53655 (10 µM) was purchased from Tocris. Insulin (0.5 µM, Sigma) was prepared from a 163 µM stock that required 0.1 M acetic acid to ensure complete dilution. TTX (1 mM) was purchased from Alomone Labs (Jerusalem, Israel). Stock solutions of these reagents were prepared in water and were stored at -20 °C and working solutions were diluted with aCSF shortly before application into the bath. The antioxidant, NAC (1 mM) (Sigma) was prepared as a stock solution in water and dissolved in patch electrode solution on the day of the experiment. Mg-ATP was included in our patch electrode solution to avoid compromising Na^+/K^+ ATPase function.

Results

Repeated high frequency stimulation of input fibers strengthens GABAergic synaptic transmission in stellate interneurons

To strengthen GABAergic transmission in stellate cells, we used repeated high frequency stimulation (HFS) of the molecular layer and used a minimal stimulation paradigm to reveal the occurrence of additional $GABA_A$ receptor-mediated IPSCs. Minimal stimulation allows recording the postsynaptic response elicited from only a few synapses activated by a weak electrical stimulus. The stimulating electrode was positioned at the upper third of the molecular layer of the cerebellum in the vicinity (50-100 µm) of the clamped interneuron. The stimulus intensity was set at amplitude that evoked 25-75% of successful responses, the rest of stimuli failed to evoke a postsynaptic response.

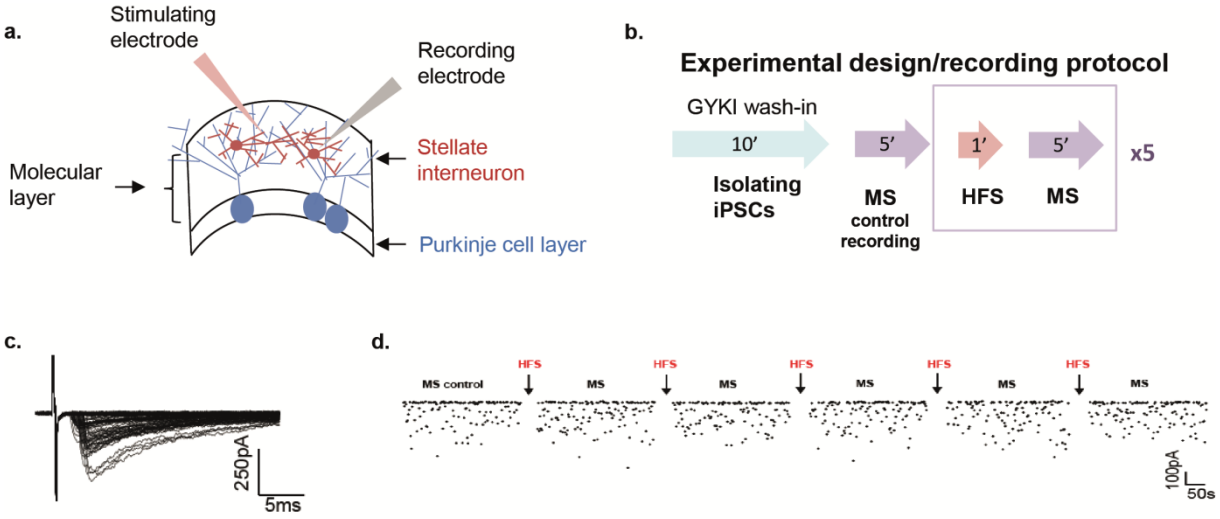

Figure 1. Experimental setup for recording evoked inhibitory events using minimal stimulation approach in combination with high-frequency stimulation.
(a) Recordings were obtained from stellate interneurons, located in the upper two thirds of the cerebellar molecular layer. Stimulating electrode was positioned 50-100µm away from the patched cell body. **(b)** 10 minutes following GYKI application, pre-HFS control MS recording was obtained, following by a series of 5 alternating HFS and MS pairs. **(c)** Example of MS recording, single sweeps recorded for 5 minutes superimposed. **(d)** Single 5 minute long MS recording windows spaced by HFS stimuli. Black dots represent the amplitudes of the responses over time.

To mimic a physiological stimulus altering the metabolic state of the cell in a way that increases mROS production, we used repeated HFS of afferents to the recorded interneuron. The stimulation protocol was applied five times interspaced by five minute long minimal stimulation

recordings (Figure 1). The percentage of transmission failures after minimal stimulation was compared at the beginning and the end of the 45 minute recording period.

Analysis of the recordings revealed that, on average, the failure rate of evoked IPSCs was significantly decreased following the series of HFSs by the end of the experiment in the majority of recorded cells (52.24 ± 5.8%, before and 25.8 ± 4.4% after the HFS, mean ± s.e.m.; n=8, *P*=0.003, paired, two-tailed Student's *t*-test) (Figure 2c). Visual inspection of the traces suggested that the majority of evoked responses by the end of the recording had amplitude smaller than 200 pA. Thus, we compared the average amplitude of this group of responses to those seen at the beginning of experiment and observed a significant increase. For instance, average amplitude of the responses at the end of the experiment normalized to the average amplitude at the beginning of experiment was 1.59 ± 0.2, (arbitrary units (a.u.), mean ± s.e.m.; n=8). Responses with amplitudes larger than 200pA did not significantly differ in the beginning and at the end of the experiment (1.03 ± 0.23, a.u., mean ± s.e.m.; n=6) (Figure 2d,e). These findings suggest that HFS strengthens GABAergic transmission by increasing the response probability to minimal stimulation.

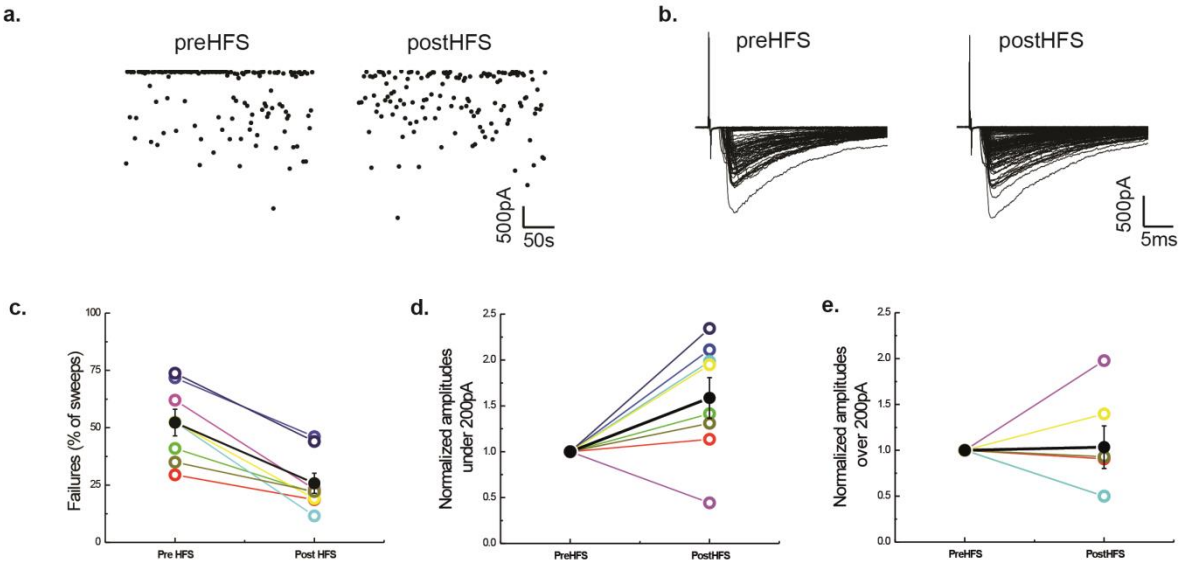

Figure 2. Increase in induced IPSCs of small amplitude at the stellate interneurons following high frequency stimulation.
(a) Evoked IPSC amplitude (-pA) recorded over time (s). Each black dot represents single evoked IPSC. Left, evoked IPSCs during the 5-minute-long control minimal stimulation (MS) recording before the HFS. Right, evoked IPSCs during the last 5 minute MS recording after the series of HSF. **(b)** Examples of raw traces of evoked IPSCs obtained during the control MS recording (left) and after the series of HFS (right). **(c)** Average amount of failures of evoking IPSCs decreased after the series of HFS. **(d,e)** Normalized average amplitude under 200 pA increased after the series of HFS **(d)** and normalized average amplitude over 200 pA did not change **(e). (c,d,e)** Open colourful circles show the data before and after HFS for single cells, Black closed circle is the average value.

Attenuating effect of NAC on the high-frequency induced strengthening of GABAergic transmission in stellate interneurons

To address the question whether the increased response probability is due to a postsynaptic mechanism and might depend on increased mROS production, the antioxidant N-Acetyl-Cysteine (NAC) was applied via the recording patch-clamp electrode. As expected based on the previous experiments in stellate interneurons (Accardi et al., 2014), NAC attenuated the effect of HFS in the recorded cells. There was no significant difference between the response failure rate before and after the series of HFS (42.9 ± 9.3%, and 44.7 ± 9.4%, respectively, mean ± s.e.m.; n=7, *P*=0.829, paired, two-tailed Student's *t*-test) (Figure 3c). Normalized amplitude under 200 pA did not change towards the end of the experiment (0.87 ± 1.6 a.u., mean ± s.e.m.; n=7) (Figure 3d). However, the average amplitude of responses larger than 200 pA was increased by the end of the recording (1.65 ± 0.27 a.u. mean ± s.e.m.; n=5) (Figure 3e).

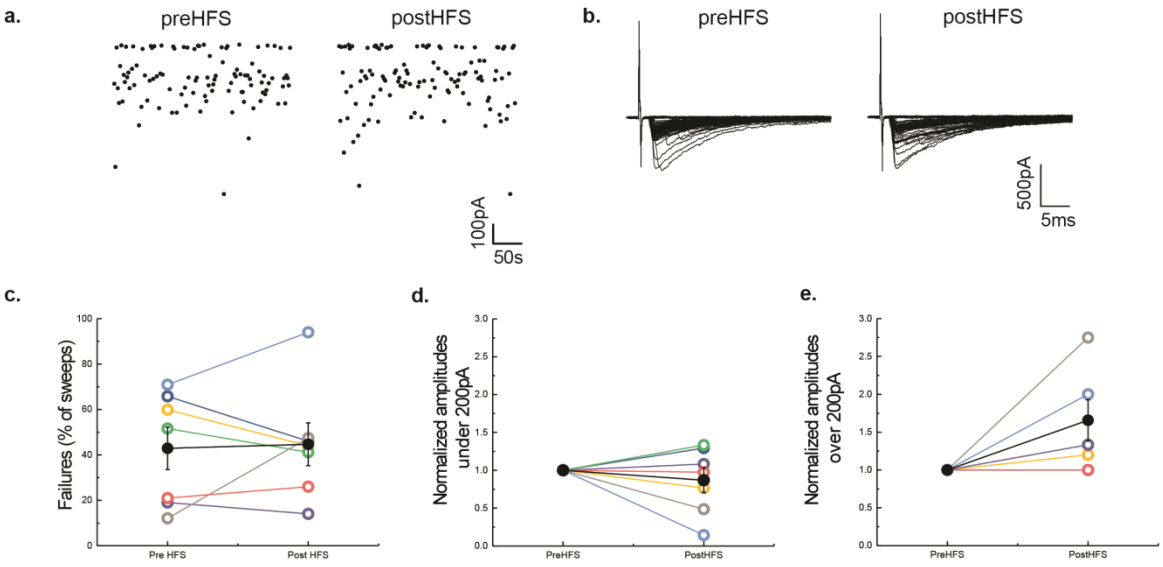

Figure 3. Attenuating effect of NAC on the high-frequency induced increase of induced IPSCs in stellate interneurons.
(a) Evoked IPSC amplitude (-pA) recorded over time (s) during the internal perfusion with 1 mM NAC. Each black dot represents single evoked IPSC. Left, evoked IPSCs during the control MS recording before the HFS. Right, evoked IPSCs during the last 5 minute MS recording after the series of HSF. **(b)** Examples of raw traces of evoked IPSCs obtained during the control MS recording (left) and after the series of HFS (right). **(c,d)** Average amount of failures of evoking IPSCs and normalized average amplitude under 200 pA did not differ before and after HFS. **(e)** Normalized average amplitude over 200 pA increased after the series of HFS. **(c,d,e)** Open colorful circles show the data before and after HFS for single cells, black closed circle is the average value.

The later effect might be due to an unrelated effect of NAC in the recorded cells. These results confirm that HFS-induced strengthening of GABAergic transmission is due, at least in large part, to a postsynaptic mechanism.

HFS promotes the occurrence of slowly decaying evoked IPSCs

α3-$GABA_A$Rs were shown to be principal mediators of GABAergic synaptic strengthening induced by mROS in stellate interneurons (Accardi et al., 2014). These receptors are characterized by their slow decay kinetics compared to α1-$GABA_A$ receptors, which represent the majority of $GABA_A$ receptors present in stellate cells. We compared the decay kinetics of the IPSCs evoked by minimal stimulation at the beginning of the recording and after the series of HFS. The decay phase of each IPSC was fitted with a single exponential function and the value was plotted in function of the event amplitude (Figure 4). HFS triggered the emergence of a population of IPSCs with slower decay kinetics. Average decay kinetics was significantly slower after the series of HFS. As seen from the distribution, the respective decay kinetics values corresponded to the events with low amplitude (Figure 4a) (12.8 ± 0.3, before and 21.2 ± 0.6 ms after the HFS; mean ± s.e.m, $P < 0.0001$, paired, two-tailed Student's *t*-test). Following application of NAC via the recording electrode, the decay kinetics of IPSCs before and after the HFS was not significantly different) (12.7 ± 0.4, before and 18.1± 0.6 ms after the HFS; mean ± s.e.m, $P=0.04$, paired, two-tailed Student's *t*-test) (Figure 4b), strongly suggesting that the effects of HFS on the induction of IPSCs with low amplitude and slow kinetics, typical of a3-$GABA_A$R-mediated events, involve mROS production.

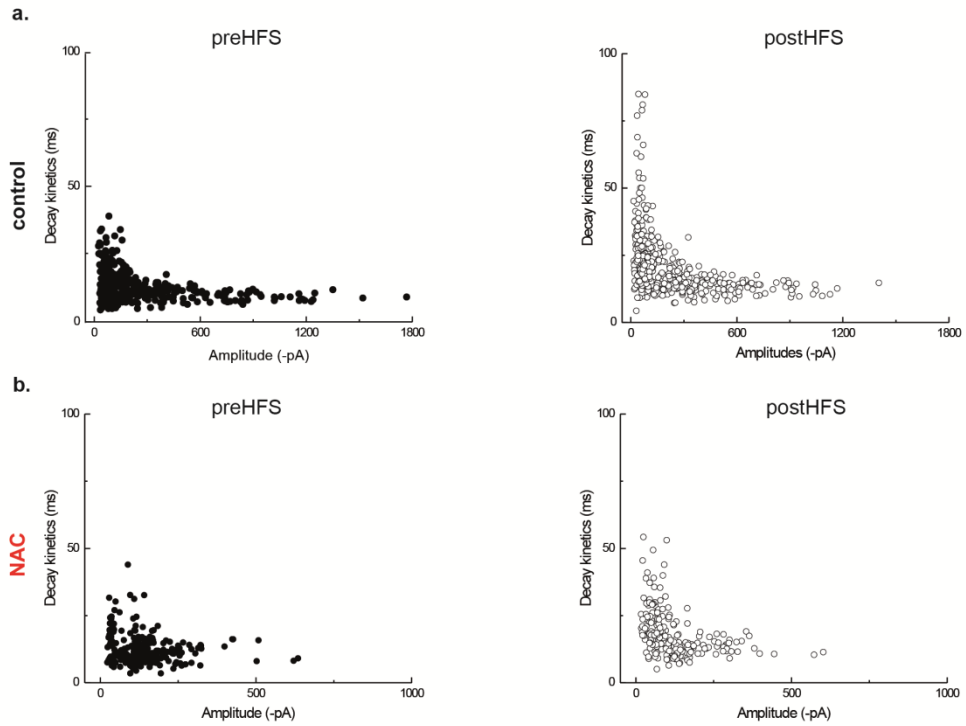

Figure 4. Slow decay kinetics of high-frequency stimulation induced IPSCs at stellate interneurons. Scatter plots of decay kinetics and evoked IPSCs amplitude before HFS (left, closed circles) and after the series of HFS (right, open circles) in **(a)** control conditions and **(b)** during the internal perfusion with 1mM NAC.

Heterogeneity of postsynaptic responses induced by repeated HFS in stellate interneurons

The majority of the recorded cells showed a decrease in response failure to minimal stimulation following repeated HFS (8 of 14).

However, the remaining six cells exhibited different responses to HFS (Figure 5). Half of them did not show a change in failure rate (51.6 ± 5.4%, before and 53.39 ± 5% after the HFS, mean ± s.e.m.; n=3, *P*=0.821, paired, two-tailed Student's *t*-test) and average amplitude (normalized average amplitude at the end of the experiment: 0.93 ± 0.1 a.u., mean ± s.e.m.; n=3) of evoked IPSCs by the end of the recording (Figure 5a). The other half showed over 2.5-fold increase in response amplitude after the first HFS (normalized average amplitude at the end of the experiment: 2.7 ± 0.7 a.u., mean ± s.e.m.; n=3). Average response failure rate decreased in the

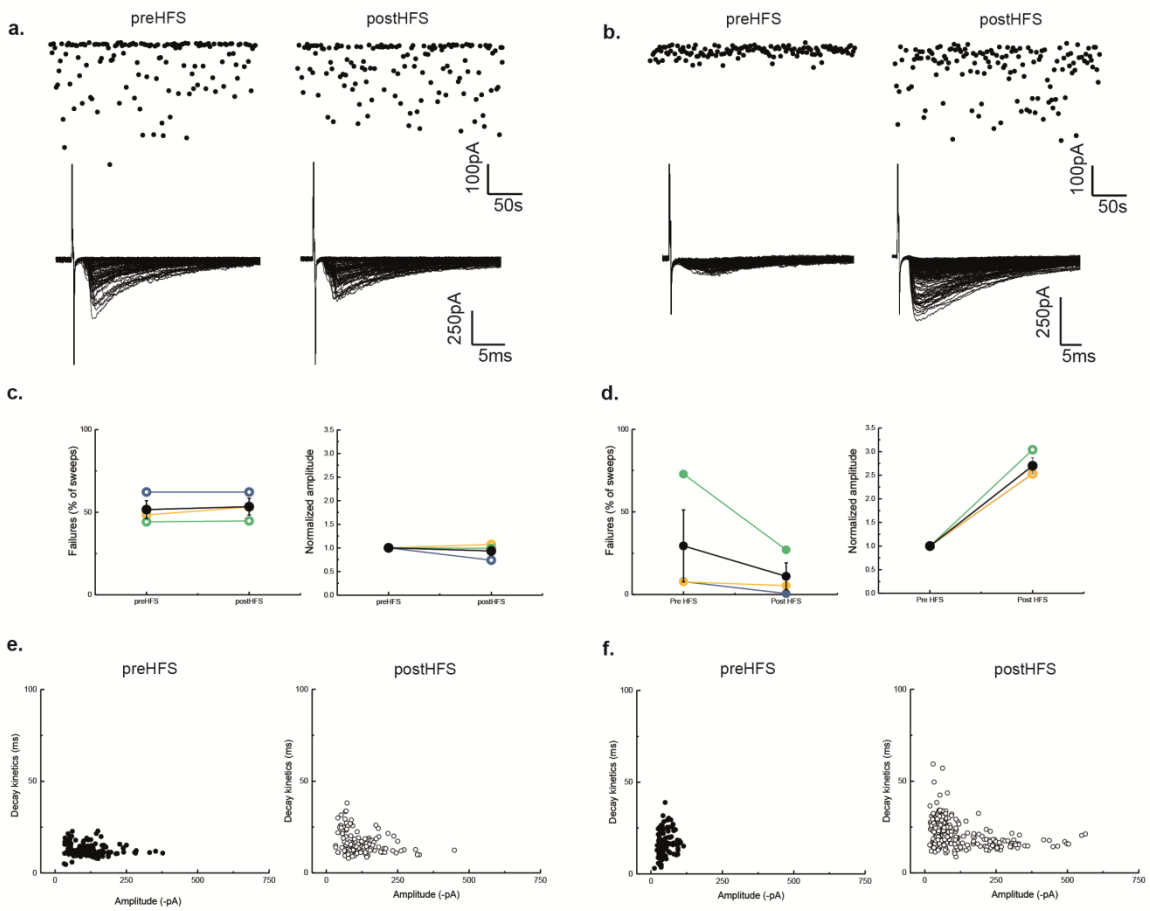

Figure 5. Different phenotypes of responses evoked by high-frequency stimulation in stellate interneurons.
(a) Example of stellate interneuron not affected by HFS. **(b)** Examples of stellate interneurons with abrupt response to HFS. **(a,b)** Upper row, evoked IPSC amplitude (-pA) recorded over time (s) before and after HFS. Lower row, raw traces of MS recording before and after HFS. **(c)** Failure rate (left) and normalized average amplitude (right) of evoked IPSCs did not change before and after HFS in the non-responding group of cells. **(d)** Normalized average amplitude (right) of evoked IPSCs significantly increased after HFS and failure rate of the evoked IPSCs (left) decreased after the HFS. **(e,f)** Decay kinetics of evoked IPSCs of non-responding (e) and abruptly responding **(f)** group of interneurons before (closed circles) and after (open circles) HFS.

course of experiment, but not significantly due to high variability within this group of recorded cells (29.4 ± 21.7%, before and 11.0 ± 8.1% after the first HFS, mean ± s.e.m.; n=3, *P*=0.471, paired, two-tailed Student's *t*-test). Difference in the effects of HFS might be related to the type of the presynaptic partner cells activated by the minimal stimulation protocol, and therefore reflect GABAergic synapse heterogeneity in the molecular layer.

Insulin induces GABAergic synapse strengthening

Insulin application was previously shown to increase cytosolic ROS in mouse cerebellum and induce GABAergic synaptic strengthening at the cerebellar granule cells (Accardi et al., 2015). We applied insulin extracellularly to explore whether it also has the potential to strengthen GABAergic synapses in stellate cells. We performed whole cell patch clamp recordings of GABAergic mIPSCs in stellate interneurons in wild-type mice under control conditions and following the application of 0.5 μM insulin into the bath solution after the first 5 minutes of recording. The cells were recorded for up to 30 minutes after the insulin wash in (Figure 6a,b,c). In the absence of insulin, there were no significant differences in the number (average counts at first 606 ± 133 and last five 599 ± 136, mean ± s.e.m.; n=8, *P*=0.887, paired, two-tailed Student's *t*-test) and distribution of mIPSCs during the first and last five minutes of recording. #numerical values of average amplitudes of distinct response populations# (Figure 6a',d).

Insulin application led to a significant increase in the number of mIPSCs at the end of the recording (average counts at first 590 ± 146 and last five 762 ± 178, mean ± s.e.m.; n=7, *P*=0.004, paired, two-tailed Student's *t*-test) emergence of a low amplitude population of mIPSCs (Figure 6b',d). Since previous research showed that mROS induced the recruitment of α3-$GABA_AR$ mediating low amplitude (and slow kinetics) mIPSCs, (Accardi, 2014) we recorded mIPSCs in stellate interneurons of α3-KO mice in the presence of insulin in the bath solution. The recordings at the beginning and at the end of the experiment showed no significant difference in the number (average counts at first 211 ± 42.5 and last five 246 ± 60, mean ± s.e.m.; n=4, *P*=0.386, paired, two-tailed Student's *t*-test) and distribution of the recorded (Figure 6c',d). Taken together, our findings suggest that postsynaptic strengthening of GABAergic transmission by insulin and HFS involve the recruitment of a3-$GABA_ARs$ via an mROS-dependent mechanism.

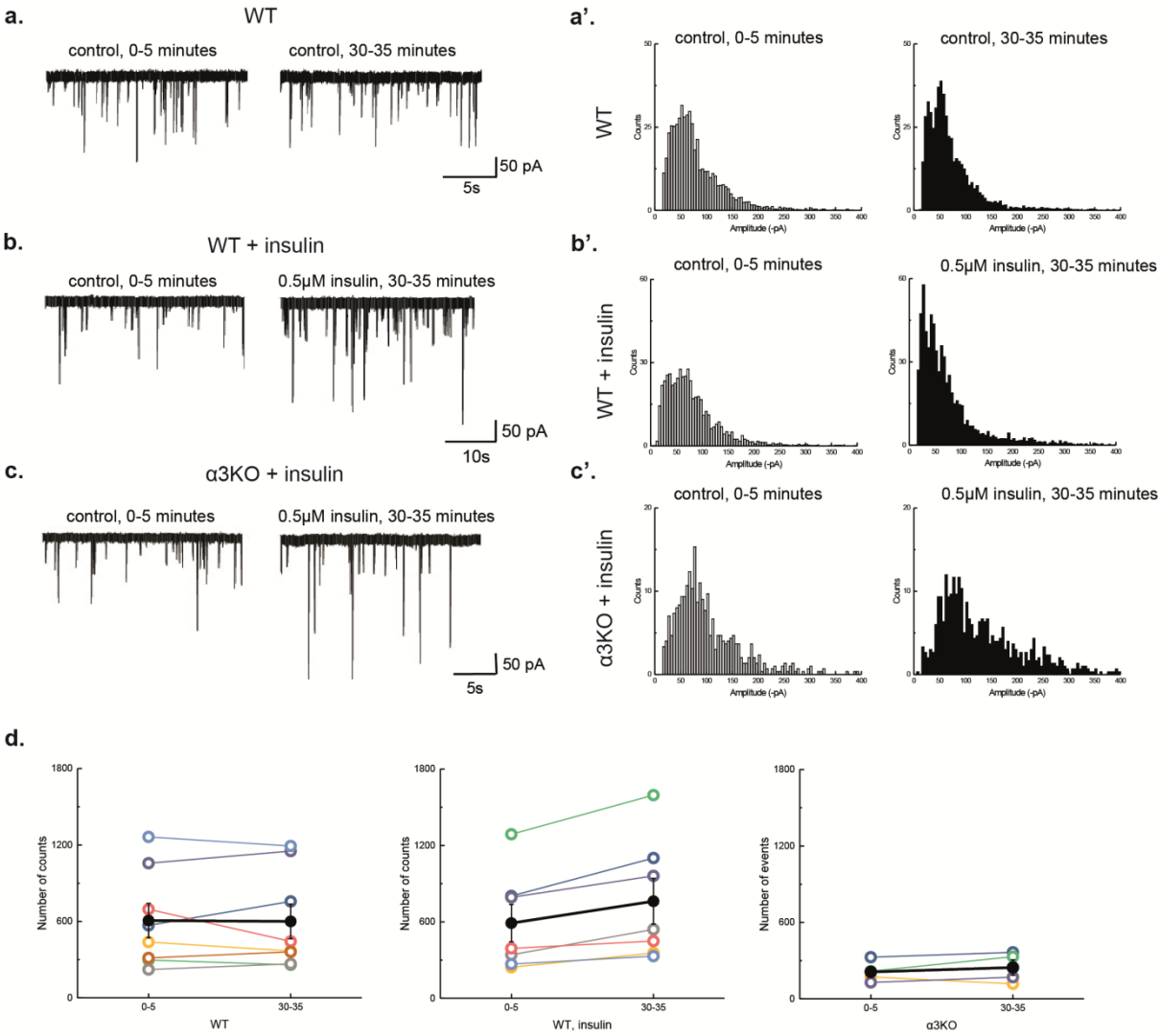

Figure 6. Insulin induces GABAergic synapse strengthening.
(a) mIPSCs recorded from the same stellate interneuron in the beginning and the end of recording period showing that frequency and amplitude are similar throughout. **(a')** Amplitude distributions of mIPSCs recorded at the beginning (0-5 minutes) and at the end (30-35 minutes) of the recording. **(b,c)** Example traces of Inhibitory events from the same interneuron at the first and last 5 minutes of recording during insulin perfusion in WT **(b)** and α3-KO mice **(c)** . **(b', c')** Amplitude histograms comparing data before and after insulin application in WT **(b')** and α3-KO mice **(c')**.

Discussion

This study advances our understanding of mROS-induced inhibitory synaptic strengthening. We showed that a pool of $GABA_A$Rs can be unsilenced under specific conditions. Because metabolic demand of a cell is reflected in mROS production, we used high frequency stimulation (HFS) to un-silence inhibitory synapses. We recorded evoked IPSCs using minimal stimulation approach before and after a series of HFS. In the majority of recordings, repeated HFS promoted an increase of frequency of evoked IPSC (Figure 2).

Events evoked by HFS have slower decay kinetics (Figure 4a), suggesting based on previous findings (Accardi et al., 2014), it is α3 containing $GABA_A$Rs that mediate this process and the effect is thus likely postsynaptic. Same setting recordings in α3-KO mice would be the next crucial experiment to confirm this conclusion.

Another piece of evidence suggesting that this mechanism of synaptic strengthening is postsynaptic is the attenuation of HFS effect by the antioxidant NAC when introduced in the recording pipette (Figure 3). NAC was previously shown to antagonize the effect of antimycin on mROS-induced increase in mIPSCs in stellate cells (Accardi et al., 2014). The decay kinetics of IPSCs pre- and post-HFS evoked events did not differ with NAC present in pipette (Figure 4b). This once more suggests a postsynaptic mechanism with mROS being an important player in the cascade of events leading to this type of inhibitory strengthening.

GABAergic synaptic strengthening can occur postsynaptically via several mechanisms. $GABA_A$R phosphorylation by multiple kinases (PKC, CaMKII, PKA, etc) can influence channel function or binding to trafficking molecules (Kittler and Moss, 2003). For example, PKC activation is known to induce dynamin-dependent endocytosis of gamma subunit containing $GABA_A$Rs (Herring et al., 2005). The number of $GABA_A$Rs on cell surface can be controlled by direct insertion of the receptors into the membrane and lateral diffusion at the synaptic membrane surface (Bogdanov et al., 2006, Bannai et al., 2009).

Using different kinase blockers in the recording pipette would be necessary to identify kinases that are involved in this pathway. It still remains unresolved whether mROS-induced strengthening occurs via lateral diffusion or direct insertion into the postsynaptic sites.

The majority of cells responded to HFS with an increase in occurrence of evoked IPSCs. There were two other smaller populations that either did not respond to the stimulation or responded that in an exaggerated manner (Figure 5). Differences in responses could be accounted for

variability in age and developmental stage of a stellate interneuron. Younger and less innervated stellate cells are located in the uppermost part of the molecular layer. It can be argued that populations of non-responding or "abruptly" responding cells could be in their maturation stages, still not being adequately integrated in the neuronal circuits. We did not find a correlation between the age of the animals and the recordings of specific types of responses. Taking this into account, we suggest that this response heterogeneity to HFS is due to the predominance of synaptic inputs from certain postsynaptic partners on possibly different subtypes of stellate interneurons or by predominant activation of presynaptic fibers originating from a specific type of presynaptic cell. Stellate cells receive inhibitory inputs from other stellate interneurons, basket, Lugaro and Golgi cells (Fritschy and Panzanelli, 2006). Specific presynaptic partners of different stellate cells could form synapses that are dominated by a certain subunit specific $GABA_A$Rs. For instance, non-responding stellate cells could represent a population of stellate interneurons or a group of activated synapses that are dominated by α1-$GABA_A$Rs. In contrast, "abruptly" responding synapses could represent a population that is dominated by α3-$GABA_A$Rs. In our setting it was not possible to distinguish the exact input fibers we stimulated during the recordings. The effect on the cells that we saw during the specific recordings could depend on the type of fibers in the vicinity of the stimulating electrode. It could also reflect the density of innervation of a specific stellate cell. Possibly, if the density of innervation is low, HFS would fail to elicit the production of mROS.

To explain the origins of this heterogeneity requires further experiments. It can be speculated that those cell where the strengthening was observed have higher levels of α3-$GABA_A$Rs expression, than those neurons that did not show any change in evoked responses. Some synapses might be lacking specific machinery that mediates mROS-induced strengthening. Previous synaptic activity could influence the amount of α3-$GABA_A$Rs filled vesicles, ready for endocytosis upon another high frequency input. To validate this assumption we need to identify presynaptic partners of stellate cells. It is not yet resolved which inhibitory inputs converge on one stellate cell. Paired (pre and postsynaptic partners recorded simultaneously) patch clamp recordings with pharmacological compounds are further planned to clarify this. It would be necessary to pharmacologically isolate specific inputs onto stellate cells. Silencing other stellate cells input with endocannabinoids (Kreitzer et al., 2002) could already reveal if there are other inhibitory inputs onto the stellate cells. Golgi cells can be fluorescently labeled, since they exclusively express glycine transporter 2 (GlyT2). Labeling would make it possible to identify Golgi cells within the granule cells layer.

We went further into looking at the triggers that through mROS elevation can strengthen inhibitory synapses at the stellate cells. Insulin was shown to elevate mROS. Previous data from cerebellar granule cells (Accardi et al., 2015) showed that insulin via increasing mROS can lead to α6-GABARs mediated synaptic strengthening. We showed an increase in small amplitude mIPSCs and the absence of this effect in α3-KO mice, proving that this strengthening is mediated by α3-$GABA_A$Rs (Figure 6).

The abundance and complexity of interaction between various types of interneurons within the cerebellar circuits is crucial in shaping the activity of Purkinje neurons that provide major cerebellar output. Inhibitory plasticity at the cerebellar interneurons fine-tunes cerebellar output with accordance to the activity within the network. mROS induced recruitment of $GABA_A$Rs to synapses may act as an internal protective feedback mechanism, activated as a result of increasing excitatory inputs, that may trigger oxidative stress. mROS-induced inhibitory strengthening is so far described in both stellate and granule interneurons in the cerebellum and it could be a universal wide-spread feedback occurring in other types of neurons in different brain regions. The dysfunctions of this mechanism could have detrimental consequences on the firing of individual neuronal subpopulations and on the overall network activity.

IV. GENERAL DISCUSSION

The overall objective of this thesis was to contribute to the understanding of the pathophysiology of neurodegenerative disorders by studying a wide range of mechanisms that could lead to them. Specifically, we investigated commonalities between Alzheimer's disease and epilepsy, which have in common an activation of the immune system, neuronal and synaptic dysfunction, and altered adult neurogenesis.

Contribution of induced seizures to AD-like pathology

In the first study, we investigated how induced epileptic activity contributes to the development of AD-like pathology in AD-susceptible brain. We explored the effects of seizures in two AD mouse models, starting with a sporadic AD mouse model. In this model, PolyI:C-induced dysregulation of the immune system during fetal stages, predisposes the mice to developing AD-like pathology after a second immune insult in adulthood ("double hit" mouse model) (Krstic et al., 2012). Our goal was to find out whether in such immune challenged mice induced chronic recurrent seizures could substitute an adult immune insult possibly through associated inflammation, and similarly lead to the development of AD-like pathological hallmarks. Next, we wanted to see whether mice that have persistent epileptic activity could develop AD-like pathology, upon an immune challenge in adulthood. Both groups of experimental animals were left to age until 9 and 15 months, before being immunohistochemically analysed for AD-like phenotypes. We did not observe any significant differences between groups of animals and controls in both experimental settings. Neither prolonged seizures in immune-challenged mice nor an immune challenge in epileptic mice aggravated AD-like pathology with ageing. These studies showed that inflammation associated with the KA lesion, persists even a year after the injection. This finding suggests that the immune responses evoked by PolyI:C might be unique in their ultimate effect on abnormal protein aggregation.

Apart from sustained microglial activation that spatially coincides with neurodegeneration in CA1, CA3 and the hilus, our group has shown that the KA-lesioned hippocampus is being populated by T lymphocytes. Furthermore, macrophage-like cells were detected in the dentate gyrus and their number positively correlated with the magnitude of granule cells dispersion. Unexpectedly, mice that lack T- and B-cells do not have a latent phase before onset of SRS and

their KA lesions are more severe due to infiltration of neutrophils. Depletion of peripheral macrophages prior to KA injection led to severe neurodegeneration of granule cells in the dentate gyrus. These findings suggest that T-lymphocytes and macrophages play a neuroprotective role in this TLE model (Zattoni et al., 2011). The mechanisms of KA-induced inflammation differs from PolyI:C challenges. Recruitment and neuroprotective action of T-lymphocytes specific for KA-induced inflammation could be one of reasons why the development of AD pathology was not observed in our experiments.

Although it is a logical conclusion from these results, it should be taken with some caution. There are a number of differences between the original "double hit" experiment (Krstic et al., 2012 (Krstic et al., 2012) and our own experiments. These differences might underlie the absence of the expected effects of PolyI:C treatment combined with chronic seizures. For instance, the mouse strain used in our experiments (C57Bl/6JOla) differs from the original C57Bl/6JRcc one. C57Bl/6JOla carries a spontaneous mutation that prevents α-synuclein expression. α-synuclein is a presynaptic protein abundant throughout the brain. α-synuclein is known for its role in Parkinson's disease (PD) pathology (Stefanis, 2012). Interestingly, α-synuclein was linked to the peripheral immune system and plays a role in some immune system related dysfunctions in PD (Kim et al., 2004). Taking into account the role of α-synuclein in the peripheral nervous system it is fair to assume that it's absence could influence the outcome of the PolyI:C challenge.

It is well known that stress caused by transportation increases the production of glucocorticoids in rodents (Landi et al., 1982). Glucocorticoids are widely used as to suppress inflammation (Cain and Cidlowski, 2017). It was shown that following transportation corticosterone can remain elevated in mice for up to 4 days (Tuli et al., 1995). For the original "double hit" study no transport was needed, since mice were bred in house (Krstic et al., 2012). For the current project pregnant dams were shipped to our animal facility at GD14 and were left to adjust to the new conditions for 3 days. It is therefore possible that mice would have needed more time to recover and acclimatize after transportation. In addition, such factors as litter effect, housing, injection procedures or handling represent additional variables that might have interfered with PolyI:C effects in our experimental setup. In the absence of a positive control that PolyI:C treatment "worked" like in the experiments from (Krstic et al., 2012), our current findings remain inconclusive.

In the second major part of this project, we tested whether chronic recurrent seizures in transgenic familial AD mice affect age-dependent AD pathology. ArcticAβ mice, which

overexpress hAPP with two FAD mutations, start developing Aβ plaques after the age of 7 months (Knobloch et al., 2007). At 2 months of age, still at pre-plaque stage, ArcticAβ mice were intracranially injected with KA to induce life-long SRS. AD mice were hypersensitive to KA and had increased mortality rate within the first 24h post-KA injection in comparison to WT controls. To prevent their immediate death during KA induced status epilepticus, animals were given a single dose of diazepam after the surgery. Although diazepam treatment prevented mortality within the first 24h, more than a half of the injected animals died within 2 months post-injection. Intracellular Aβ levels are already increased at the age when mice receive KA injection. This fact suggests that the hypersensitivity to KA might be due to the early pre-plaque AD-related pathology. Brain tissue of the KA-injected ArcticAβ mice that survived 6 months after KA injection had a "mild" KA-lesion phenotype with usually only CA1 degeneration in the ipsilateral dorsal hippocampus. In comparison, only a few WT mice also had "mild" lesions indicating some degree of variability in the effects of KA, probably due to technical reasons. Therefore, it is conceivable that the ArcticAβ mice that died during ageing had more severe lesions causing a fatal status epilepticus. We did not investigate here the causes of the increased susceptibility of ArcticAβ mice to seizures, because this was part of a separate research project in our laboratory.

In ArcticAβ mice, Aβ oligomers start to accumulate intraneuronally at the age of 3 months. Progressive accumulation continues with ageing to peak between 7 and 15 months. The first plaques, extracellularly and around blood vessels appear at the age of about 6 months. At 3 months, the transgenic animals have increased pain sensitivity, as detected in the hotplate test, and strong locomotor and exploratory hyperactivity. This hyperactivity disappears with age. Starting after the age of 6 months, ArcticAβ develop anxiety and start performing significantly worse in an array of cognitive tests in comparison to their WT littermates (Knobloch et al., 2007). Our results show that increased sensitivity to seizure-inducing agents precedes cognitive impairments in ArcticAβ mice and coincides with the beginning of progressive accumulation of intracellular Aβ oligomers. Intraneuronal soluble Aβ oligomers are suggested to be more neurotoxic than insoluble forms. Intracellular Aβ oligomers were shown to be responsible for synaptic dysfunction, as they are often found associated with atypical dendrites with abnormally stacked membranes and non-functional cytoskeletal organelles (Takahashi et al., 2002, Oddo et al., 2003, Umeda et al., 2011). Aβ-related synaptic dysfunction that occurs early in this transgenic model could contribute to the increased sensitivity to KA.

To estimate the effect of induced seizures on the development of AD pathology we quantified the amount of Aβ plaques in 9 months-old KA- or vehicle-treated ArcticAβ mice. Contrary to our original expectations, the KA-lesioned ArcticAβ animals had reduced plaque pathology. In particular, in comparison to NaCl-injected ArcticAβ controls, KA-lesioned mice had significantly fewer plaques in the hippocampus. There was a significantly smaller number of Aβ plaques in the ipsilateral vs contralateral KA-injected hippocampus. A tendency towards decreased plaque load was observed in the neocortex of KA-injected vs NaCl-injected transgenic mice.

Subcortical innervation was previously hypothesized to be involved in the initial seeding of toxic Aβ species (Braak and Del Tredici, 2013). The hippocampal formation receives extensive noradrenergic and serotonergic innervation from the locus coeruleus and upper raphe nuclei respectively. It turned out that the KA lesion does not lead to atrophy of these monoaminergic inputs in the hippocampus of ArcticAβ mice. Therefore, the reduced plaque pathology likely had another origin.

Interestingly, at the age of 9 months, a sustained microgliosis was still present in the ipsilateral hippocampus in the WT KA-lesioned mice, but was restricted only to the degenerated CA1 region in the KA-injected transgenic AD mice. The consequences of the KA insult, such as neurodegeneration, sustained immune response and SRS seem to prevent or delay the formation of Aβ plaques locally. The plaque load in the neocortex was not significantly different between KA-and NaCl-injected AD mice, which suggests that the group of the animals that survived until the age of 9 months was not simply consisting of the animals with reduced "baseline" plaque pathology. However, it would be interesting to look at the extent of the AD-like pathology at the later time points to see whether the formation of plaques in this group of animals is temporally delayed.

Reduced gamma rhythm was reported in several FAD mouse models (Stam et al., 2002, Palop et al., 2007, Verret et al., 2012b, Gillespie et al., 2016). Recently, it was shown that induction of fast-spiking parvalbumin-positive interneurons at gamma (40Hz) frequency decreased production of Aβ and triggered changes in gene expression in microglia, that results in its "engulfing" state (Iaccarino et al., 2016). It could be speculated that the reduced amount of Aβ plaques in our model is due to prolonged increased network activity that includes oscillations in gamma range as a component.

In fact, it was shown that gamma frequency oscillations (30–80 Hz) are present during the chronic period in the KA rodent model and that they actually precede the occurrence of spontaneous seizures (Bragin et al., 2005). "Mild" KA lesion phenotype in ArcticAβ mice implies preservation of CA3 region. Gamma activity occurs in the hippocampal CA3 in mice with KA-induced chronic seizures and the discharge frequency of oriens lacunosum-moleculare (OLM) interneurons changes from theta to gamma frequency band in this epileptic mice model (Dugladze et al., 2007). Bath application of KA onto brain slices of WT mice can induce gamma oscillations in CA3 (Hajos et al., 2000, Lu et al., 2011). It was suggested that alterations in intrinsic firing properties of interneurons underlie changes in the rhythmogenesis.

Neurogenesis in AD predisposed brain

In an attempt to explain sensitivity of ArcticAβ mice to KA, we characterized their pattern of adult neurogenesis. Abnormal adult neurogenesis affects seizure susceptibility in some of epilepsy mouse models. Suppression of adult neurogenesis increases the effect of KA in mice (Iyengar et al., 2015). Contrary to this finding, it was also shown that reduced adult neurogenesis reduces seizures in the pilocarpine mouse model of TLE. In AD mouse models, adult neurogenesis is frequently affected (Sun et al., 2009, Biscaro et al., 2012, Bonds et al., 2015). BrdU labeling in ArcticAβ mice revealed higher proliferation rates, but decreased survival of progenitor cells in the hippocampal DG. eGFP viral labeling of newborn neurons showed decreased dendritic arborization and decreased spine density in immature adult born neurons of transgenic mice. ArcticAβ mouse line is among those FAD models that overexpress mutant human APP. Those mouse models tend to have increased NPC proliferation, which can be related to the presence of high quantities of soluble APPα (sAPPα), a proteolyte of APP cleavage by α-secretase. sAPPα is a proliferation factor of adult progenitor cells (Demars et al., 2011, Demars et al., 2013). Early upregulation of intracellular, potentially harmful, Aβ species could be the reason for delayed maturation of the adult born neurons.

Although ArcticAβ mice had increased NPC proliferation in comparison to WT littermates, the survival of these cells was decreased and there was no difference in cell fate of NPCs. Eventually this "equalized" the number of newborn neurons between the genotypes. Impaired maturation of newborn neurons could potentially lead to impaired development and integration within the network. It was shown that adult-born neurons have a net inhibitory effect on the DG-CA3 network (Acsady et al., 1998). Apart from affecting cognitive performance, weakening this effect on networks within the hippocampus could contribute to high sensitivity of ArcticAβ to KA.

Inhibitory transmission in neurodegenerative disorders

GABAergic interneurons comprise a diverse family of cells that can play an important role in pathophysiological mechanisms of both AD and epilepsy. Their basic biological function is to control and synchronize the activity of principal excitatory neurons and other interneurons, by which they participate in sustaining oscillatory network activity and network synchrony (Buzsaki (Buzsaki and Draguhn, 2004, Kepecs and Fishell, 2014). Numerous subtypes of inhibitory GABAergic interneurons control network synchrony and oscillatory brain rhythms (Buzsaki and Draguhn, 2004). Some interneurons fire rather tonically, independent of brain states (Sohal et al., 2009, Lapray et al., 2012). Abnormalities in interneurons have been reported for many brain dysrhythmias and cognitive dysfunctions associated with various neurological conditions (Verret et al., 2012b, Hunt et al., 2013, Southwell et al., 2014, Tong et al., 2014). Cognitive deficits typical for AD, mechanisms of which rely on interneuron function, are described in TLE patients (Jokeit and Ebner, 1999, Sinforiani et al., 2003, Ito et al., 2009b). Altered gamma oscillation has been described in AD patients and in AD mouse models (Palop et al., 2007, Verret et al., 2012b, Gillespie et al., 2016). Parvalbumin (PV)-positive interneurons (Lapray et al., 2012, Verret et al., 2012a, Kemere et al., 2013).

In the second major part of the thesis, we focused on investigating a mechanism of synaptic strengthening recently described in cerebellar stellate interneurons. In the cerebellum, stellate cells inhibit Purkinje cells on their dendrites and by this contribute to controlling the action potential firing rate of Purkinje neurons. Specifically, it was shown that mitochondrial-derived reactive oxygen species (mROS) do not only contribute to negative effects of oxidative stress, but actually have a physiological signaling function. mROS elevation leads to strengthening of inhibitory transmission in stellate cells by recruiting a population of $GABA_A$Rs to the synapse, which differ in subunit composition compared to the resident $GABA_A$Rs (Accardi, 2014). Cerebellar stellate cells mostly express α1 subunit-containing $GABA_A$Rs. Increase in intracellular mROS triggers emergence of postsynaptic events with unique kinetic properties, that in stellate cells correspond to α3 subunit-containing $GABA_A$Rs. Increase in mROS production is a reflection of cellular metabolism. Stellate interneurons use mROS as a distinctive indicator of the need to decrease firing in a response to a stimulus that could potentially lead to the oxidative stress. mROS-dependent inhibitory synaptic strengthening was also shown in cerebellar granule cells. Elevation of mROS triggered recruitment of α6 subunit-containing $GABA_A$Rs and as in case of stellate cells, had no effect on resident α1 subunit-containing

$GABA_A$Rs. Interestingly, insulin probably by elevating mROS also triggers this strengthening, but the underlying signaling cascade activated by insulin remains to be determined.

Cerebellar granule cells are excitatory glutamatergic neurons, originating from a germinal zone distinct from stellate cells,. The fact that mROS-dependent synaptic strengthening is present in these two quite distinct cell types suggests that it might be an important common protective self-feedback mechanism against oxidative stress possibly present in many other CNS neurons. One of the next steps would be to confirm this possibility with patch-clamp recordings in neurons in other brain areas, such as for instance the hippocampus. Breakdown or deterioration of such an important protective mechanism could be involved in many neurological disorders.

This project focused on studying further this unique inhibitory plasticity. We drew parallels between glutamatergic silence synapses and mROS-dependent plasticity. We used minimal stimulation experimental approach to reveal un-silencing of inhibitory synapses. Before and after challenging a stellate cell with high frequency input stimulus we recorded evoked IPSCs. In most of the cases after high frequency stimulation a new population of IPSCs emerged among the evoked events. Increase in frequency of those events points towards an increase of the presynaptic release probability. This effect was prevented by an antioxidant NAC present in the patch pipette, suggesting this plasticity is postsynaptic and mROS dependent. Slow kinetics of the newly emerged responses suggested it is α3-$GABA_A$Rs mediated. Further crucial experiment is the confirmation of absence of this plasticity in α3-KO mice.

Some of the cells that were recorded responded to series of HFS with either no increase in frequency of evoked events or with a drastic 2.5 fold IPSCs increase. We hypothesize that the difference of responses depends on the origin of fibers that were stimulated each time. Stellate cells receive inhibitory inputs from other stellate and basket cells, Lugaro, globular and Golgi cells (Fritschy and Panzanelli, 2014). We suggest that subunit specificity of GABA synapse depends on the identity of the presynaptic partner. For example, synapses where we did not observe increase of frequency of evoked events could represent α1-$GABA_A$Rs predominant stellate to stellate type of connection and those with abrupt increase type of responses could be α3-$GABA_A$Rs Golgi to stellate type of input. To prove this, patch clamp recordings of stellate-stellate or Golgi-stellate pairs are planned.

For finding future therapeutic targets it is important to dissect specific pathways and mechanisms of mROS-induced plasticity. For other types of inhibitory plasticity, multiple possible postsynaptic mechanisms were demonstrated (Gaiarsa et al., 2002). Regulated vesicular

insertion, or lateral diffusion of the receptors at the synaptic membrane surface (Bannai et al., 2009) interference with channel properties via phosphorylation of receptors by multiple kinases (PKC, CAMKII, PKA) (Kittler and Moss, 2003). Gephyrin is a major scaffolding protein at inhibitory synapses. ROS signaling molecule nitric oxide (NO) can lead to S-nitrosylation of gephyrin. This can lead to a reduction of putative inhibitory synapses and weakening of the synaptic strength (Dejanovic and Schwarz, 2014). Taking this into account, we suggest that in mROS-dependent strengthening, gamma-aminobutyric acid receptor-associated protein (GABARAP), could play an important role. Using peptides that block interaction between the receptors and gephyrin or GABARAP would be important to understand scaffolding mechanisms behind this type of plasticity.

We wanted to see whether other stimuli that potentially elevate mROS are capable of inducing mROS-dependent plasticity in stellate cells. Insulin was previously shown to elevate ROS through its receptor (Goldstein et al., 2005). Bath application of insulin has already been shown to induce inhibitory strengthening in cerebellar granule cells (Accardi et al., 2015). We went on to test whether this holds true for stellate cells. We recorded TTX insensitive miniature IPSCs (mIPSCs) before and after bath application of insulin and saw an increase of the events with slow decay kinetics. This was not observed in α3-KO mice, which again suggests that this plasticity is mediated by α3 - and not α1-$GABA_A$Rs.

Stimuli such as high frequency stimulation or insulin application in excess can induce drastic oxidative stress. mROS-dependent plasticity can be viewed as a protective internal feedback mechanism, activation of which causes inhibition and thus decreases metabolic need of the cell, preventing aggravation of oxidative stress. Mitochondrial dysfunction and oxidative stress are described in many neurodegenerative disorders, including AD (Islam, 2017, Mamelak, 2017). If mROS-dependent strengthening is indeed a universal mechanism throughout the brain it is fair to assume that hindering it will lead to abnormal neuronal firing and thus network dysfunction. Fine-tuning of interneuronal firing can be achieved through heterogeneity of $GABA_A$Rs recruited to the synapses in response to overwhelming stimuli.

In many neurological disorders, including AD and epilepsy, network activity responsible for cognition and memory encoding is altered already at pre-clinical stages (Sperling et al., 2009, Anticevic et al., 2012, Fahoum et al., 2013, Fahoum et al., 2014, Oser et al., 2014, Seidman et al., 2014). The alterations include activation and deactivation deficits, abnormal network oscillations and network hypersynchrony. In people predisposed to AD, network alterations occur decades before the diagnosis (Filippini et al., 2009, Bateman et al., 2012, Reiman et al.,

2012). In healthy individuals during cognitive tasks that require attention, fMRI signals in specific brain areas are increased (for example hippocampus during memory encoding). So called default-mode networks (DMN) are activated during inwardly oriented network activity, such as daydreaming. During attention demanding tasks DMNs are deactivated (Raichle et al., 2001, Boyatzis et al., 2014). Default networks are comprised of functionally distinct association areas (precuneus/posterior cingulate cortex, medial prefrontal cortex, medial, lateral and inferior parietal cortex) that interact with each other and with the medial temporal lobe (Greicius et al., 2003, Greicius et al., 2004, Vincent et al., 2006, Buckner et al., 2008, Buckner et al., 2009). Interestingly, amyloid deposition measured by PiB-PET (Pittsburgh compound B-positron emission tomography – is used to image amyloid deposits in AD diagnosis) mostly accumulates in cortical regions involved in the default network (Klunk et al., 2004, Buckner et al., 2005) and these cortical areas have reduction of metabolism, functional disruption, and atrophy in early stages of clinically diagnosed AD and mild cognitive impairment (MCI) (Greicius et al., 2004, Rombouts et al., 2005, Celone et al., 2006, Sorg et al., 2007, Pihlajamaki et al., 2008). Impaired deactivation of DMNs during attention demanding tasks is observed in many pathological conditions (Sperling et al., 2009, Anticevic et al., 2012, Fahoum et al., 2013, Oser et al., 2014, Seidman et al., 2014). Before the emergence of the first obvious cognitive symptoms, individuals with cerebral amyloid deposits show hyperactivation of hippocampus (probably a compensation for early cognitive deterioration) and decreased DMN deactivation. Similar synchrony disturbances are observed in pre-symptomatic APOE ε4 (major risk factor for AD) gene allele carriers, FAD mutations carriers and patients with mild cognitive impairment (MCI) that eventually develop AD (Bookheimer et al., 2000, Dickerson et al., 2005, Celone et al., 2006, Trivedi et al., 2008a, Trivedi et al., 2008b, Filippini et al., 2009, Quiroz et al., 2010, Bakker et al., 2012, Reiman et al., 2012, Sepulveda-Falla et al., 2012, Kunz et al., 2015).

Interestingly, disturbances in functional connectivity of DMN spatially overlap with amyloid deposits in AD patients (Hedden et al., 2009, Mormino et al., 2011, Mormino et al., 2012, Dennis and Thompson, 2014). Based on studies in primates, it has been suggested that DMN would exhibit less gamma power during execution of attention-demanding tasks than during resting baseline periods (Harris and Thiele, 2011). Precise mechanisms on cellular and network levels of the DMN generation are still not identified, but interneurons undoubtedly play an important role in this highly orchestrated synchronous activity. Various impairments in interneuron firing or synaptic function and plasticity could underlie impaired synchrony and deactivation of DMNs. Persistent activity in the DMN areas could trigger increased Aβ

production, which in turn would deteriorate synaptic function. mROS induced plasticity could potentially be a common mechanism of inhibitory strengthening in various interneurons and principal cells. This hypothesis has to be further investigated. Dysfunction of this plasticity could lead to network abnormalities and excessive oxidative stress, which could lead to extensive neuronal loss. mROS induced plasticity is a promising candidate for search of novel therapeutic targets.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AD,PD	Alzheimer's disease, Parkinson's disease
ADAM	a disintegrin and metalloproteinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APH-1	anterior pharynx-defective 1
APOE	apolipoprotein E
APP	amyloid precursor protein
BACE	beta-secretase
BBB	blood brain barrier
BrdU	bromodeoxyuridine
CAA	cerebral amyloid angiopathy
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CD68	cluster of differentiation 68
ChAT	choline acetyltransferase
CNS	central nervous system
DMN	default mode network
EEG	electroencephalogram
EPSC	excitatory postsynaptic current
GABA	gamma-aminobutyric acid
GABARAP	gamma-aminobutyric acid receptor associated protein
GD14/17	gestational day 14/17
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GSK3-β	glycogen synthase kinase 3 beta
HFS	high frequency stimulation
IPSC	inhibitory postsynaptic current
KA	kainic acid
LMW/HMW	low molecular weight/ high molecular weight
LPS	lipopolysaccharide
LTD	long-term depression
LTP	long-term potentiation
MCI	mild cognitive impairment

mGluR	metabotropic glutamate receptor
mROS	mitochondrial reactive oxygen species
NAC	N-Acetyl-Cysteine
NFT	neurofibrillary tangles
NMDA	N-Methyl-D-aspartate
NPC	neural precursor cells
NSAID	nonsteroidal anti-inflammatory drug
PEN2	presenilin enhancer 2
PET	positron emission tomography
PKA	protein kinase A
PKC	protein kinase C
PKCα/PICK1	protein kinase C α/ protein interacting with C kinase 1
PolyI:C	polyinosinic:polycytidylic acid
PS1, PS2	presenelin 1, presenelin 2
SRS	spontaneous recurrent seizures
SVZ	subventricular zone
TH	tyrosine hydroxylase
TLE	temporal lobe epilepsy
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha

Hippocampal formation:

CA1	cornu ammonis 1
sp	stratum pyramidale
sr	stratum radiatum
slm	stratum lacunosum-moleculare
CA3	cornu ammonis 3
sl	stratum lucidum
sp	stratum pyramidale
DG	dentate gyrus
ml	molecular layer
gcl	granular cell layer

CURRICULUM VITAE

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PUBLICATIONS

ORIGINAL ARTICLES

A tale of two retinal domains: near-optimal sampling of achromatic contrasts in natural scenes through asymmetric photoreceptor distribution. Baden T., Schubert T., Chang L., Wei T., **Zaichuk M.**, Wissinger B., Euler T. **Neuron**2013

Distinct and common functions of mTORC1 and mTORC2 in Purkinje cells. Angliker N., Burri M., **Zaichuk M**., Fritschy JM., Rüegg M.A., **EJN** 2015

Contribution of early Alzheimer-related pathophysiology to the development of acquired epilepsy. Gschwind T., Lafourcade C., **Zaichuk M**., Gfeller T., Rambousek L., Knuesel I., Fritschy JM. submitted

Effects of acquired seizures on AD-like pathology in a mouse model of familial Alzheimer's disease. **Zaichuk M**., Schwerdel C., Fritschy JM. to be submitted

Mechanisms of inhibitory synaptic strengthening at cerebellar stellate interneurons. **Zaichuk M**., Larson E., Fritschy JM., Bowie D. in preparation

Various gain-of-function effects in SCN2A-mutations. Lauxmann S., Verbeek N.E., Liu Y., **Zaichuk M**., Mueller S., Lemke J., Lerche H., van Kempen M.J.A., Hedrich U.B.S. in preparation

ABSTRACTS FOR POSTERS

Role of adult neurogenesis in increased sensitivity of ArcticAβ mice to kainic acid. **Zaichuk M**., Gschwind T., Knuesel I., Fritschy JM. Poster presentation, **Society for Neuroscience Annual Meeting**, San Diego, USA, 2016

Effects of acquired seizures on Alzheimer's disease (AD)-like pathology in mouse models of familial and sporadic AD. **Zaichuk M**., Gschwind T., Knuesel I., Fritschy JM. Poster presentation, **9th FENS Forum of Neuroscience**, Milan, Italy, 2014

Induced seizures in familial and sporadic AD. **Zaichuk M**., Gschwind T., Knüsel I. and Fritschy JM. **ZNZ Symposium, Zürich, Switzerland 2014**

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